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SOME THERMODYNAMIC AND KINETIC ASPECTS OF METABOLIC PHOSPHORYLATION

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DURING THE LAST FIFTEEN YEARS, the part played by phosphate in cellular processes has been made clear: phosphate is the means by which a large part at least of the energy of catabolic processes is made available for adaptive processes in the cell. The immediate source of energy for these adaptive processes is believed to be unstable ('energy rich') phosphate compounds e.g., creatine phosphate and adenosine triphosphate). These are formed from inorganic phosphate at the expense of the free energy of degradation of carbohydrate and probably of other intermediates.

The chemistry and some thermodynamic aspects of reactions involving phosphorylation have been reviewed by Barron (4, 5), Kalckar (26, 27), Meyerhof (46) and Lipmann (40–42). We have attempted a rather more thorough use of the thermodynamic approach under the following heads:

I. A general examination of the use that can be made of thermodynamic data and a consideration of what additional information, especially kinetic, is needed for a full understanding of cellular processes.

II. An examination of the thermodynamic information concerning phosphorylation brought about by anaerobic glycolysis.

III. An examination of the thermodynamic information concerning phosphorylation brought about by oxidation of carbohydrate, together with a critical study of some of the experimental data on the amount of phosphorylation that is produced.

IV. An examination of the possible functions of intermediary carriers in the transport of hydrogen and of phosphate.

V. An examination of some problems of kinetics that arise from these considerations.

It must be pointed out that the thermodynamic argument applied in sections II and III is unlikely to lead to incorrect results provided that the assumptions made and the numerical values used are accurate. The assumptions made have been clearly indicated in the text, and the experimental figures are unlikely to be far wrong; for this reason the figures given in these sections should be of value for comparison with experimental results. On the other hand much of the argument used in the last two sections is based on kinetic considerations of a fairly simple nature. Since almost nothing is known of the actual kinetic conditions within the cell, it is uncertain how far the results obtained can be rigidly applied to the cell. For this reason, and in view of the much greater un-

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certainty in the assumptions made and the numerical values used, the figures deduced in the last two sections are to be taken more as illustrative of the type of calculation which must eventually be made than as of immediate use for comparison with experiment.

Finally we have suggested what sort of experimental evidence is needed to solve the problems that have been raised.

I. GENERAL

Only in the case of the anaerobic breakdown of carbohydrate is the exact nature of the process known by which inorganic phosphate is transferred to phosphate acceptors; however, the mechanism can reasonably be supposed to be general and no evidence opposes this supposition.

The type of changes involved may be expressed as follows: *a*) An intermediate R forms a compound with inorganic phosphate whose free energy of hydrolysis is relatively low, $R + P \rightarrow RP$; *b*) the compound RP then undergoes a reaction (such as dehydrogenation or decarboxylation), $RP \rightarrow R' P$, with the formation of a product which is relatively unstable thermodynamically, that is, of high free energy of hydrolysis; and *c*) R′ P reacts with an acceptor A (such as creatine or adenylic acid), $R' P + A \rightarrow R' + AP$.

In order that such a series of changes may occur in any given state of concentrations of reactants and products, it is a necessary condition that the total change of free energy shall not be positive. The total change of free energy may be regarded as the sum of the changes of free energy involved in the processes $R \rightarrow R'$ (which will be negative) and $A + P \rightarrow AP$ (which will be positive).

It is convenient to classify reactions according to their *standard free energy*, ΔF_0. This is usually defined as the change of free energy when the activity of each reactant and product is unity. (To simplify further argument we shall take the activity as being measured by the molar concentration.) ΔF_0 is related to the conventional equilibrium constant of the reaction by $\Delta F_0 = -RT \ln K$ (where ln represents $\log_e$). However, where the reactants and products have concentrations other than 1 molar, the actual free energy change is given by

$$\Delta F = \Delta F_0 + RT \ln \frac{\text{Product of concentrations of products}}{\text{Product of concentrations of reactants}}.$$

Thus, in order to predict whether a given reaction $R \rightarrow R'$ can lead to the phosphorylation of a given acceptor, it is necessary to know not only the respective values of ΔF_0 but also the concentrations of all the substances concerned. A conclusion based on the values of ΔF_0 alone might be seriously wrong.

Usually the change $R \rightarrow R'$ occurs not as an isolated reaction but as one stage of a sequence of reactions such as

$$Q \xrightarrow[\Delta F_{01}]{} R \xrightarrow[\Delta F_{02}]{} R' \xrightarrow[\Delta F_{03}]{} S$$

and the concentrations of R and R′ will then be partly determined by the preceding and succeeding reactions. Suppose that only the reaction $R \rightarrow R'$ leads to phosphorylation of A and cannot occur unless A accepts phosphate, that the free energy of $A + P \rightarrow AP$, under the conditions chosen, is $\Delta F'$, and that the concentrations of

R and R′ are not such that ΔF_2 is negative enough for phosphorylation of A to occur (i.e., $\Delta F_2 + \Delta F' > 0$). Then R may accumulate as a result of conversion of Q to R, and R′ may become depleted by conversion to S. ΔF_2 thus becomes more negative and, if this can go far enough, a point is reached at which phosphorylation can occur. However, ΔF_2 can reach a sufficiently negative value only if the overall free energy change of Q → S is large enough and negative. This depends on the overall change of standard free energy and on the concentrations of Q and S. Thus for phosphorylation to occur

$$\Delta F_{01} + \Delta F_{02} + \Delta F_{03} + RT \ln [S]/[Q] + \Delta F' < 0 \qquad 1$$

must be satisfied. This condition may also be stated in the form

$$\Delta F_{02} + RT \ln [R']/[R] + \Delta F' < 0. \qquad 2$$

The whole of the free energy of the reaction Q → S will be available for phosphorylation (at the stage R → R′) when the reactions Q → R and R′ → S are at equilibrium (and therefore involve no changes of free energy). In this situation the maximum amount of energy is made available at a single stage.

If the reaction R → R′ can occur without phosphorylation of A, then the concentrations of R and R′ reached, when a steady state is attained, will depend on the rates of this and of the other reactions. In this case, a purely thermodynamic argument is not enough to answer the question whether the reaction Q → S can lead to the phosphorylation of A, since the ratio [R′]/[R] might never reach a low enough value even if *equation 1* were satisfied. Therefore, kinetic data are also required.

It will not be found in practice that any system can transfer energy with an efficiency of 1. This could be so only in the absence of any side reaction, and if all stages were at equilibrium, when the rate of reaction would be infinitely slow. The rate may also be insufficient, even if the total free energy is sufficiently negative, if the concentration of any intermediate should fall too low. Thus, in the example given, if ΔF_{02} is too positive, the value of [R′]/[R] needed to satisfy *equation 2* may be very small; consequently, the low concentration of R′ may become a rate-limiting factor.

In general, knowledge of cellular processes must be based, in the first place, on information about the chemical nature of the substances and reactions concerned. Thermodynamic information next enables limits to be set to the possible development of free energy by reactions and thence to the possibilities of coupling between them. But knowledge of the standard free energies of reactions is not enough for this purpose; the actual free energy changes are needed and this involves knowledge of the concentrations of all the substances concerned. This introduces the necessity for kinetic information, to set limits to the concentrations of reacting substances consistent with the rates at which individual and overall reactions occur. Ultimately, the aim is to be able to account for cellular reactions, both in the steady state and when the steady state is disturbed in any particular way.

At present, naturally, chemical information is most complete; thermodynamic information is nearly complete in some fields but incomplete in others; kinetic information which is relevant to conditions within the cell is largely lacking.

II. PHOSPHORYLATION ASSOCIATED WITH ANAEROBIC GLYCOLYSIS

Lundsgaard (45) showed that phosphorylation of creatine occurs in whole muscle following a short tetanus. In his most favored experiments (his table 7, experiments 7–11) the molar ratio of creatine phosphate (CrP) formed to lactate (HL) formed varied between 1.6 and 1.9. Meyerhof and Lohmann (48) and Lehnartz (39) further showed that phosphorylation of creatine accompanies anaerobic glycolysis in muscle brei and press juice, but they did not show an exact proportionality between the two processes. Lehmann (38) showed a quantitative phosphorylation of adenylic acid by 2-phosphopyruvate (PyP) and of creatine by adenosine triphosphate (ATP).

This and later work has made it clear that adenylic acid or adenosine diphosphate (ADP) accepts phosphate (P) at two stages of the glycolytic process: *1*) from 1:3 diphosphoglyceric acid (diPG acid; Needham and Pillai, 51; Bücher, 11; Meyerhof *et al.*, 49); *2*) from 2-phosphopyruvic acid (PyP) (Lehmann, 38, Lardy and Ziegler, 36). In turn, Banga (2) confirmed that *3*) adenosine diphosphate and adenosine triphosphate can transfer phosphate to creatine. Phosphate is acquired by the glycolytic system at three stages: *4a*) glycogen, through phosphorolysis by inorganic phosphate, gives glucose-1-phosphate (G-1P) or *4b*) glucose, with phosphate from ATP, gives glucose-6-phosphate (G-6-P); *5*) fructose-6-phosphate (F-6-P), with ATP, gives fructose 1-6-diphosphate (HDP); *6*) 3-phosphoglyceraldehyde (PGA), with inorganic phosphate, after dehydrogenation forms 1-3-diphosphoglyceric acid.

The stoichiometry of these stages does not seem to be in doubt. It appears, therefore, that the maximum ratio of moles of phosphate combined to moles of lactate formed is 1.5 in the case of glycogen and 1 in the case of glucose. This appears to be in conflict with the results of Lundsgaard quoted above, but close examination of his data shows that the discrepancy can be accounted for. Thus, while after a one-minute recovery the inorganic phosphate had returned nearly to its value in resting muscle (13 to 15 millimolar), the creatine phosphate (at 14 to 17 millimolar) was still considerably below its resting concentration (17 to 24 millimolar). Furthermore, the decrease of inorganic phosphate (4.5 to 8.5 millimolar) is greater than the increase of creatine phosphate in the same experiments (4.5 to 5.5 millimolar). These results can be explained if there is a fall in the concentration of hexose diphosphate during the recovery, leading to a ratio of CrP/HL greater than 1.5, with a simultaneous increase in the concentration of hexose monophosphates; this would cause a decrease of inorganic phosphate greater than the increase of creatine phosphate. These considerations illustrate the difficulties of dealing quantitatively with changes in a complex system.

It is interesting to consider the stoichiometrical ratio of 1.5 in the light of the thermodynamic data, now very nearly complete, which are set out in table 1. Column 1 gives the index number of the reaction; column 2 gives the reaction (in certain cases main reactions are dissected into simpler reactions which are indexed by letters); column 3 gives the reference to the literature; columns 4 and 5 give the experimental values of K or of E'_0, whichever has been measured; column 6a gives the values of ΔF_0, per $\frac{1}{2}$ mole of glucose for those reactions which sum to glycogen → lactate; and column 6b gives the values of ΔF_0 for the other reactions. All values

are for pH 7 to 7.5 and for 37°C. At the bottom of the table are the summed values of ΔF_0 for the two main overall reactions.

The following points require special comment:

Reaction 4a. This is the only reaction in the series for which no exact data are available. It is known that fructose-6-phosphate is not phosphorylated by inorganic

TABLE 1

1	2	3	4	5	6a	6b
		REF. NO.	K	E'_0	ΔF_0 cal/ ½ mol. of glucose	
1	Glycogen + P → G-1-P	15	0.42		+270	
2	G-1-P → G-6-P	69	15.7		−840	
3	G-6-P → F-6-P	69	0.43		+260	
4	F-6-P + ATP → HDP + ADP					−5,100
a	F-6-P + P → HDP	1	10^{-2}		+1,400	
b	ATP → ADP + P	42				−6,500
5	HDP → PDHA + PGA	22	1.2×10^{-4}		+2,800	
6	PDHA → PGA	47	2.8×10^{-2}		+1,100	
7	PGA + P + Co → diPGAcid + CoH_2	74	0.9		+70	
a	PGA → PGAcid + H_2	2		−0.67		−18,100
b	H_2 + Co → CoH_2	8		−0.28		
c	PGAcid + P → diPGAcid	2				+18,200
8	diPGAcid + ADP → 3-PGAcid + ATP	2	5.1×10^3			−5,200
a	diPGAcid → 3-PGAcid + P	2			−18,200	
b	P + ADP → ATP	42				+13,000
9	3-PGAcid → 2-PGAcid	50	0.11		+1,340	
10	2-PGAcid → PyP	44	3.6		−790	
11	PyP + ADP → Py + ATP	36				−3,000
a	PyP → Py + P				−16,000	
b	P + ADP → ATP	42				+13,000
12	Py + CoH_2 → HL + Co				−4,600	
a	Py + H_2 → HL	6		−0.18		
b	CoH_2 → Co + H_2	8		−0.28		
13	ATP + Cr → ADP + CrP	2	2.3×10^{-3}			+3,700
a	ATP → ADP + P	42				−13,000
b	Cr + P → CrP					+16,700
	½ glucose (glycogen) → HL				−33,200	
	2 Cr + 2P → 2 CrP				+33,400	

[1] Value assumed; see text. [2] Values deduced; see text.

phosphate to a detectable extent, whereas ATP leaves no detectable amount of fructose-6-phosphate unphosphorylated. Taking the limit of detectability as 1 per cent, the value of the standard free energy of phosphorylation of fructose-6-phosphate must lie between $-2.303 \log_{10} 10^{-2}$, that is +2800 calories/mole, and +13,000 minus 2800, that is +10,200 calories/mole. We have taken the lower limit (see below), which agrees with Lipmann's (40) estimate for a 'low-energy' phosphate ester.

Reaction 8. Bücher (11) appears to have made a direct study of this equilibrium, but we have been unable to find any publication of the quantitative results. Fortunately, it has been possible to obtain information about this equilibrium indirectly through the measurement of *reaction 7* by Warburg and Christian (74) of the reaction $\frac{1}{2}$ HDP + CoI + P + ADP = PGAcid + $CoIH_2$ + ATP by Meyerhof *et al.* (49) and from *reactions 5* and *6*.

For *reaction 7* Warburg and Christian (74) obtained the values of K given in table 2.[2] From the data of Meyerhof *et al.* (their table 5), using the equilibrium constants for *reactions 5* and *6*, it is possible to calculate values of K for *reaction 8* (table 3). Using Lipmann's (42) value of −13,000 calories for ΔF_0 of hydrolysis of ATP, a value of −18,200 calories for ΔF_0 of hydrolysis of diphosphoglyceric acid is obtained (*reaction 8a*). Using this value and −0.28 volt for E'_0 of CoI, the value of E'_0 for phosphoglyceraldehyde-3-phosphoglyceric acid (*reaction 7a*) is −0.67 volt.

TABLE 2

$$K = \frac{[\text{diPGAcid}]\,[CoH_2]}{[\text{PGA}]\,[P]\,[Co]}$$

[P] millimolar	K
0.87	0.99
16.8	0.87
33.6	0.88

log. mean K = 0.91

TABLE 3

$$K = \frac{[\text{PGAcid}]\,[\text{ADP}]}{[\text{diPGAcid}]\,[\text{ADP}]}$$

EXPERIMENT[1]	K × 10^{-3}	EXPERIMENT[1]	K × 10^{-3}
1a	2.5	2	3.3
1b	9.7	3	8.2

log. mean K = 5.1 × 10^3

[1] Meyerhof *et al* (49), table V.

Reaction 13. Banga (2) measured the equilibrium constant of *reaction 13*. ΔF_0 for *reaction 13b* is thence obtained using Lipmann's value of ΔF_0 for the hydrolysis of ATP.

Discussion. Table 1 shows that, under standard conditions, the conversion of glycogen to lactate supplies just insufficient energy to phosphorylate 2 moles of creatine per mole of lactate; alternatively, that at equilibrium there will be less creatine phosphate than creatine, if the concentrations of glycogen, lactate and phosphate are all unit. Correction for the actual stationary concentrations of initial and end products is applied, taking the concentrations (in resting muscle)

[2] In a paper which appeared after the manuscript of this review was complete, Meyerhof and Oesper, *J. Biol. Chem.* 170: 1, 1947, describe extensive measurements on this and related equilibria; their value of K for reaction 7 at pH 7 is close to that of Warburg and Christian. We cannot agree with Meyerhof and Oesper that Warburg and Christian did not regard this reaction as an equilibrium; indeed, they specifically stated that it is so and their quantitative results (table 2) support this assertion.

of phosphate and of lactate as 14 and 2 millimolar, respectively. We neglect the effect of concentration of glycogen on the free energy, for the effect is small *per residue of glucose* since glycogen is a high polymer. Since at equilibrium the total free energy change of the reaction

$$\tfrac{1}{2} \text{ glycogen} + 2\ \text{Cr} + 2\ \text{P} = \text{HL} + 2\ \text{CrP}$$

is zero, then

$$-33{,}200 + 33{,}400 + 2.303\ \text{RT}\ (\log [\text{HL}] - \log [\text{P}] + 2 \log [\text{CrP}]/[\text{Cr}]) = 0$$

whence

$$[\text{CrP}]/[\text{Cr}] = 2.3.$$

This agrees reasonably with the values found for resting frog muscle (18) under anaerobic conditions.

In this calculation we have assumed that fructose-6-phosphate is phosphorylated by inorganic phosphate at 14 millimolar; in fact, it receives its phosphate only from ATP. This corresponds to a net phosphorylation of 1.5 moles of creatine instead of 2 and would lead to a much higher predicted value for [CrP]/[Cr]. This discrepancy would, however, largely disappear if it were assumed that the free energy of phosphorylation of fructose-6-phosphate had the higher value (see above). On the other hand, if its value is about +2800 calories/mole (the value assumed above), the reaction with ATP must be far from equilibrium; otherwise, in resting muscle at least one of the following would have to hold: very low ATP, very low phosphate, very high HDP or *reactions 1*, *2* and *3* very far from equilibrium, none of which appears to be true. Our assumption of the lower value is consistent with that made in explaining why Lundsgaard obtained ratios CrP/HL greater than 1.5. This assumption of the lower value implies a 'loss' of most of the free energy of ATP breakdown at this stage and an overall efficiency of about 0.75.

It is to be noticed also that while ΔF_0 for *reaction 8a* is sufficient to phosphorylate creatine to a [CrP]/[Cr] ratio greater than 1, ΔF_0 for *reaction 11a* is insufficient. This must mean that the stationary ratio of [PyP]/[Py] must be considerably greater than 1. The high thermal efficiency estimated in the whole muscle (45) means that there is little margin of energy and that all reactions, except *4*, *8a* and *11a*, must be near their equilibria.

III. PHOSPHORYLATION ASSOCIATED WITH THE OXIDATION OF CARBOHYDRATE

The information available here is in every way less complete than in the anaerobic case. There is no doubt that phosphorylation of creatine does occur during the aerobic recovery of whole muscle, associated with the oxidation of glycogen, but there seem to be no reliable estimates of the ratio of phosphorylation to oxidation. For example, different methods, thermal and chemical, give a wide range of values for the Meyerhof Quotient; Lipmann (40) accepts the value of 6, but though this is near the mean value, no great reliance is to be placed upon it. Closer study of phosphorylation in tissue preparations has necessarily awaited the chemical definition of the path of oxidation of glycogen; this can now be taken as established, at

least in its main features (31). Little work has been done on the case corresponding to glycolysis, namely the oxidation of glycogen to pyruvate, and no clear evidence has been obtained on the degree of phosphorylation connected with this process.

However, various measurements of oxidative phosphorylation accompanying the oxidation of pyruvate and its intermediates have been made, chiefly by Cori and collaborators, by Ochoa and by Tzibakowa and Belitzer.

If we take the Meyerhof Quotient as being not less than 5, then the number of moles of phosphate combined, per $\frac{1}{2}$ mole of glucose oxidized, must be at least 5×1.5; allowing 1.5 for the contribution of the process glycogen → pyruvate (as in the anaerobic reaction) this leaves six to be accounted for by the oxidation of pyruvate. This is a minimum estimate and evidently the P:O ratio (56) must be greater than 1 in its average value and probably considerably higher in some of the individual stages of the oxidation. Since in none of the work of Cori and collaborators is a P:O ratio as great as 1 found, we shall confine attention to the work of Ochoa. (The work of Belitzer and Tzibakowa (7) is not directly available to us.)

Free Energy of Oxidation of Pyruvate. We shall first calculate the total free energy derivable from the oxidation of pyruvate, in order to find the limit of possible values for the P:O ratio. The heat of combustion of dry glycogen is given as 4238 calories/gram by Kharasch (29) or 686,600 calories/glucoside residue. The entropy of glycogen per glucoside residue may be estimated as 35.4 cals/°C. from the value of 50.7 for glucose less 15.3, the average decrease found for several reactions in which water is eliminated (75). Then for the complete oxidation $(C_6H_{10}O_5) + 6\ O_2 = 6\ CO_2 + 5\ H_2O$, $\Delta S = +62.7$ calories/°C. (using the values for O_2, CO_2 and H_2O given by Parks and Huffman, 61) and $\Delta F = \Delta H - T\Delta S = -686{,}600 - 298 \times 62.7 = -705{,}300$ calories per glucoside residue at 25°C. (O_2 and CO_2 at one atmosphere and liquid H_2O). Now

$$\left\{\frac{\partial\left(\frac{\Delta F}{T}\right)}{\partial T}\right\}_p = \frac{-\Delta H}{T^2}.$$

Therefore, $\Delta F_0(37°) = -704{,}900$ calories/glucoside residue.

The standard free energies of formation of lactate from glycogen and of the oxidation of lactate to pyruvate (at 37°C. and pH 7) are already known (table 1), and the standard free energy of formation of H_2O is obtained from Parks and Huffman (61). By difference, the standard free energy of oxidation of pyruvate is obtained (table 4). This value is for molar pyruvate at 37°C. and pH 7, O_2 and CO_2 at one atmosphere and liquid H_2O.

It is convenient to correct this value to the conditions used in experiments, namely pyruvate 0.025 M and CO_2 at 40 mm. Hg (these being of the order of magnitude of those in the cell). Furthermore, the pressure of O_2 may be less than one atmosphere. Applying these corrections we obtain:

$$\Delta F/\text{atom of oxygen} = -55{,}500 - 705 \log_{10} p(O_2) \text{ calories} \qquad 3$$

where $p(O_2)$ is the partial pressure of the O_2 in atmospheres.

The standard free energy calculated here is for the oxidation of glycogen in the

dry state, but the free energy of solution of glycogen, per glucose residue, is certain to be relatively small and may be neglected. The value of ΔF for the oxidation of pyruvate is also affected by the value assumed for ΔF_0 of *reaction 4a* of table 1; however, at the worst, the value given for ΔF per atom of oxygen would be 700 calories too positive. This difference also is unimportant.

Several interesting conclusions can be drawn at once from these results:

a) The value of the free energy depends on the partial pressure of oxygen at which the oxidation effectively occurs. Now a given pressure of oxygen is related to a potential on the redox scale by the equation for an oxygen electrode

$$E'_h = 0.82 + \frac{2.303RT}{4F} \log p(O_2) = 0.82 + 0.0153 \log p(O_2) \qquad 4$$

where F is the electrochemical equivalent and where E'_h is measured against a hydrogen electrode at pH 7. We can then, by means of *equations 3* and *4*, calculate the free energy available from the oxidation of pyruvate at any value of E'_h. If we take the standard free energy of phosphorylation of creatine as +16,700 calories/mole, and assume that phosphorylation occurs at a [CrP]/[Cr] ratio of unity, we can calculate from these free energies the maximum P:O ratio obtainable from the oxidation of pyruvate at any E'_h, that is, at any effective pressure of oxygen that we choose. Some examples are given in table 5.

Evidently a P:O ratio of about 3 is the greatest possible, even if atmospheric oxygen exerts its full oxidizing power. It seems unlikely, however, that it can do so in the cellular system (see below). The next lower known redox system is cytochrome C, whose E'_0 is +0.26 volt (67); if oxidation occurred effectively at this potential, a P:O ratio of only about 1.8 could be obtained. Both these values assume an efficiency of 1.

b) The standard free energy (37°C., solid glycogen, pyruvate 1 M, pH 7, $p(O_2) = p(CO_2) = 1$ atmos.) available from the aerobic conversion of glycogen to pyruvate is obtained from table 4. Per atom of oxygen it is $\frac{1}{2}(-704{,}900 + 548{,}600) = -78{,}300$ calories. This is far in excess of the minimum needed to form 1.5 or even 2 moles of creatine phosphate at a [CrP]/[Cr] ratio of unity.

c) Calculation of the free energy of formation of glycogen from its elements, from the heat of combustion and entropy of glycogen and from the known free energies of formation of CO_2 and H_2O, gives −142,200 calories per glucose residue. Thence for the reaction glycogen + H_2O = glucose, $\Delta F_0 = -18{,}500$ calories per mole of glucose. (The values of the free energies of formation of water and of glucose were taken from Parks and Huffman, 61). This value may be wrong by several thousand calories, but it makes it clear why glucose must be phosphorylated by ATP, in contrast with glycogen which can react with inorganic phosphate.

P:O Ratio. In view of the conclusions reached in section *a*) above, a close examination of Ochoa's results becomes necessary.

a) *Brain dispersions* (56), to which were added pyruvate, fumarate, phosphate, magnesium, coenzyme I and adenylic acid, with creatine, glucose or hexosemonophosphate as phosphate acceptor and fluoride to hinder the hydrolysis of ATP, showed some phosphorylation of adenylic acid or of creatine; the phosphorylation

was greater with glucose or hexosemonophosphate as acceptors. The P:O ratio fell rapidly with time from initial values near 2. Lipmann (42) has suggested the extrapolation of Ochoa's data to a P:O ratio greater than 2 at zero time, but this seems to be very risky.

b) Heart muscle preparations were studied under similar conditions (57). With these, the P:O ratio did not fall with time, and the same P:O ratio was found whether glucose or creatine was the phosphate acceptor. The values averaged 1.91 with glucose and 1.88 with creatine, with considerable variation about these values in different experiments. Fluoride was again included.

It was shown in separate experiments that fluoride retarded the liberation of phosphate from added ATP and increased the extent of phosphorylation of glucose by added ATP, though considerable liberation of inorganic phosphate still occurred. Ochoa argued that adenosine triphosphatase (ATP-ase) was still partly active, even in the presence of 0.025 M fluoride (at which concentration its maximum effect is obtained), and that this lowers the P:O ratio. He attempted, therefore, to allow

TABLE 4

REACTION	ΔF_0 (37°) *cal/mol. of glucose*
1. Glycogen + $6O_2$ → $6CO_2$ + $5H_2O$	−704,940
2. Glycogen + H_2O → 2 HL	−66,400
3. 2 HL → 2 Py + $2H_2$	+23,000
4. $2H_2$ + O_2 → $2H_2O$	−113,120
5. (1 − 2 − 3 − 4) 2 Py + $5O_2$ → $6CO_2$ + $4H_2O$	−548,620

TABLE 5

E'h *volt*	−ΔF *cal/atom of oxygen*	MAXIMUM P:O	E'h *volt*	−ΔF *cal/atom of oxygen*	MAXIMUM P:O
+0.82	55,500	3.3	+0.26	29,700	1.8
+0.70	50,100	3.0	−0.02	16,700	1.0
+0.34	33,400	2.0			

for this assumed lowering by measuring the P:O ratio obtained in the same system during the anaerobic reaction hexosediphosphate to phosphoglyceric acid. Now Needham and Pillai (51) and Meyerhof *et al.* (49) had demonstrated in similar preparations, which did not contain ATP-ase, a P:O ratio of about 1 for this reaction; Ochoa obtained P:O ratios of about 0.6. He therefore applied a 40 per cent proportionate correction to the aerobic values, obtaining corrected values for the P:O ratio of about 3.

We do not accept the validity of this corrected value for the following reasons. Ochoa's results for the liberation of phosphate from ATP added to the preparation, in the absence of fluoride and in the presence of 0.025 M fluoride, are plotted in figure 1. Although he does not quote an analysis of his sample of ATP, it is clear from his data that the content of labile phosphate is near to the value required and that all the labile phosphate is removed by enzymes in the preparation. Hydrolysis in the presence of fluoride follows a first-order course closely (full line in fig. 1). Hydrolysis in the absence of fluoride also follows approximately a first-order course

with the same velocity constant as in the presence of fluoride (full line in fig. 1), but a closer inspection of the data shows that the rate of liberation of phosphate is nearly the same in the two cases after five minutes and that the uninhibited reaction is therefore a compound one. The rapid component of this compound reaction (complete within five minutes) is inhibited by fluoride, while the slower component is unaffected. Moreover, the end point of the reaction in the presence of fluoride is at 60 per cent of that of the uninhibited process.

These facts exclude both the possibility that fluoride produces a *partial* inhibition of adenosine triphosphatase, since this could not give a first-order process going to a 60 per cent end point, and also the possibility that fluoride inhibits the breakdown of adenosine diphosphate, since this could explain neither the inhibition of the faster component reaction nor the 60 per cent end point. On the other hand, ATP-ase

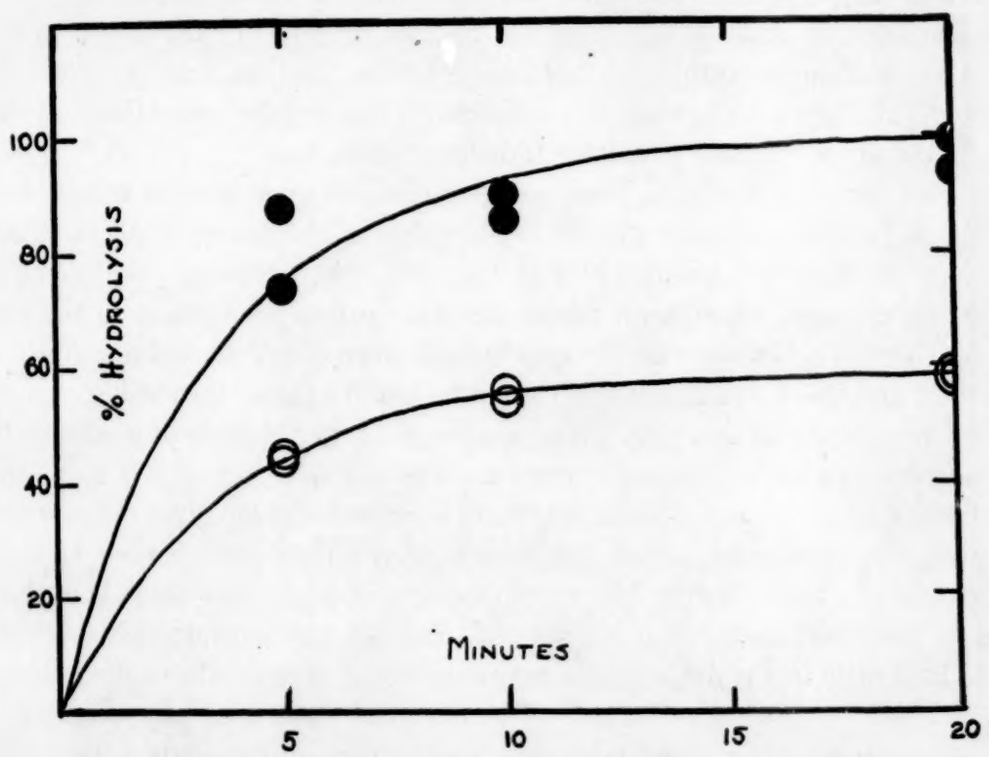

Fig. 1. FILLED CIRCLES: without NaF; open circles: 0.025 M NaF

must be inhibited by fluoride because fluoride is shown to bring about an absolute increase in the amount of phosphate transferred from added ATP to glucose and a great increase in the aerobic phosphorylation of glucose; inhibition of the breakdown of ADP would not account for either of these facts since ADP does not phosphorylate glucose (14). We are forced to conclude, therefore, that 0.025 M fluoride produces a complete inhibition of adenosine triphosphatase and that the phosphate which is liberated from added 'ATP' in the presence of fluoride is not derived from ATP.

This implies that Ochoa's sample of ATP, though it contained the theoretical amount of labile phosphate, consisted of only 40 per cent of true ATP, the residual 60 per cent being another material of identical composition. Although this conclusion is a surprising one, it is supported by Bailey's (1) finding that solid barium-ATP may break down to a considerable extent on long storage, mostly into adenylic

acid and pyrophosphate; while Lohmann (43) showed that frog muscle containing a pyrophosphatase is not inhibited by fluoride at 38°. The actions of ATP-ase on ATP (40 per cent inhibited by fluoride) and of pyrophosphatase on pyrophosphate (60 per cent, not inhibited by fluoride) would account for Ochoa's results.

This interpretation of Ochoa's results also explains the fact that the same P:O ratio was obtained for the phosphorylation of both glucose and creatine. It would be difficult to understand this finding if adenosine triphosphatase were still residually active in the presence of 0.025 M fluoride, since the great difference of the free energies of phosphorylation of the two acceptors would demand very different ratios of [ATP]/[ADP] to effect their phosphorylation; one would have expected appreciably lower values of P:O for creatine than for glucose. The single experimental objection to our interpretation is Ochoa's value of only 0.6 for the P:O ratio of the anaerobic reaction in the presence of fluoride. We are unable to explain this.

We consider, in spite of this, that the balance of evidence favors the conclusion that 0.025 M fluoride completely inhibits ATP-ase, and we conclude that Ochoa's observed P:O ratios for the aerobic reaction do not require correction but measure directly the uncomplicated oxidative transfer of phosphate.

c) Ochoa (58) observed the phosphorylation which accompanies the oxidation of α-ketoglutarate in the heart muscle preparation in the presence of malonate and fluoride. He obtained similar P:O ratios with α-ketoglutarate and pyruvate in parallel experiments, though all the values were rather lower than in the previous work. There is no reason why the conclusions given above should not apply to this work also, and the P:O ratios should again be taken at their face values.

These results, as we interpret them, suggest that, per .5 mole of glucose oxidized, the number of moles of phosphate combined is about 11 ($1.5 + 5 \times 1.9$), assuming a contribution of 1.5 moles of phosphate from the aerobic stage glycogen → pyruvate. Assuming the same yield from the anaerobic reaction glycogen → lactate, this corresponds to a value for the Meyerhof Quotient of 7.3. This value is in the upper range of direct estimates of this quotient, whereas any considerably higher value for the P:O ratio in the oxidation of pyruvate would give a value outside the range.

Mechanisms for Phosphorylation. The essential nature of any reaction which leads to phosphorylation has been indicated. The intermediate with which the phosphate first combines must be capable of undergoing a reaction whose change of free energy is negative and which leads to a phosphate compound of lower stability. Such a reaction could be a dehydrogenation, a dehydration, a hydration or a decarboxylation. Some light may be thrown on oxidative phosphorylation by an examination of the supposed stages of oxidation of pyruvate from this viewpoint; at the same time we shall examine the thermodynamic data. The data at present available are given in table 6 (columns as in table 1).

The whole series of reactions involves 5 dehydrogenation, 3 decarboxylation and 3 hydration or dehydration stages. If it is supposed that each of these could be mechanistically concerned in the transfer of 1 phosphate, the total is 11; if the hydration and dehydration stages are excluded (no experiment suggests otherwise), the total is 8. For comparison with this, if we assume oxidation at +0.26 volt, the total free energy change during the oxidation of molar pyruvate would allow for

phosphorylation of 8.8 moles of creatine per mole of pyruvate, at a [Cr]/[CrP] ratio of unity. It is interesting to note that the data available on individual stages show that the sum of the free energies of reactions 7, 8, 9 and 11 is −32,600 calories and of reactions 3, 4, 5 and 10 is −35,600 calories, each being approximately sufficient for the synthesis of 2 moles of CrP. Experimentally it appears that the P:O ratios for *reactions 7* and for *9* plus *11* are not greater than 1 (12, 13, 25), and Ochoa (57) has

TABLE 6

	2	3	4	5	6	
					ΔF_0 = cal/mol. CO_2 = 40 mm.; other substances molar	
		REF. NO.	K	E'_0	*a.* O_2 = 1 atmos. E'_h = +0.82 volt	*b.* E'_h = +0.26 volt
1	Pyruvate + oxaloacetate → condensation product + H_2O		—	—		
2	Condensation product + O → cis-aconitate + CO_2		—	—		
3	Cis-aconitate + H_2O → iso-citrate	24	4		−850	−850
4	Iso-citrate + CoII → oxalosuccinate + H_2CoII (pH7: 22°C)	59, 60	3.3		−730	−730
5	oxalosuccinate → α-ketoglutarate + CO_2 (pH7: 22°C)	59, 60	2.3×10^3 (CO_2 molar)		−8970	−8970
6	α-ketoglutarate + O → succinate + CO_2 + H_2O		—	—		
7	Succinate + O → fumarate + H_2O	37, 9		0	−37,900	−12,000
8	Fumarate + H_2O → malate	33	3.2		−720	−720
9	Malate + CoI → oxaloacetate + H_2CoII	35		−0.17	+5,080	+5,080
10	H_2CoII + O → CoII + H_2O	[1]		−0.28	−50,800	−25,000
11	H_2CoI + O → CoI + H_2O	8		−0.28	−50,800	−25,000
	Accounted for				−145,690	−68,190
	Balance (reactions 1, 2, 6)				−131,810	−79,910
	Total				−277,500	−148,100

[1] Value assumed.

shown that the oxidation of H_2CoI (*reaction 11*), added to a heart muscle preparation, does not cause phosphorylation.

Now, if the phosphate transferred is more than 8 moles per mole of pyruvate (a P:O ratio greater than 1.6), then, excluding the hydration and dehydration reactions, one or more single stages must lead to the transfer of more than one phosphate each. This could happen in two ways: the substrate molecule could combine with two or more molecules of phosphate, all of which are made less stable by a subsequent reaction (though no experimental evidence supports the existence

of such compounds) or (as suggested by Lipmann, 41) an intermediate carrier of hydrogen might be involved, itself undergoing an oxidation-reduction and phosphorylation-dephosphorylation cycle. Such a carrier mechanism raises certain specific problems which will be discussed in the next section. A more general kinetic problem, however, is raised by both mechanisms and this will be discussed in section 5.

IV. CARRIERS

General. Carriers have hitherto been considered almost exclusively with respect to their function in the transport of hydrogen. A carrier is a substance which can accept hydrogen from a reducing system and pass it on to an oxidizing system; it may form an essential or a permissive link in the process of hydrogen transport. Generally, the carrier will have a different stationary ratio of oxidized to reduced forms at these two sites, maintained by the oxidative and reductive processes at them, and by diffusion between them. This difference of ratios corresponds to a difference of E'_h and could be the means by which a part of the energy of the oxidative process is made available. The maximum energy obtainable from such a process is $\Delta F = -2F\Delta E'_h = -RT\Delta(\ln[Ox]/[Red])$ calories per mole of H_2 transferred, where $\Delta E'_h$ is the difference between the values of E'_h at the two sites. (For a value of ΔF of $-16{,}700$ calories, $\Delta(\log_{10}(Ox)/(Red))$ is about 12.)

Transfer of phosphate could result from reactions of a carrier molecule C by the following cycle of processes:

$CH_2 + P \rightarrow CH_2P$	'low energy phosphate'
$CH_2P - 2H \rightarrow CP$	'high energy phosphate'
$CP + A \rightarrow C + AP$	phosphorylation of acceptor
$C + 2H \rightarrow CH_2$	reduction of unphosphorylated carrier

This differs from the general mechanism of phosphorylation by a substrate in being cyclic. Thus, while it is the unphosphorylated C which is hydrogenated, it is the phosphorylated CH_2P which is dehydrogenated, implying a difference in the enzymatic processes of reduction and oxidation of the carrier. It must be assumed either that there are two different enzymes or, equally possible, that there is a single enzyme having a double specificity. Furthermore, since there is no net change in the amounts of oxidized and reduced carrier, the energy is not derived from oxidation of the carrier, but comes, indirectly, from the oxidation of the ultimate reductant (substrate) by the ultimate oxidant (effectively oxygen, cytochrome, flavoprotein etc., according to the conditions).

In principle, any carrier system could perform a transfer of phosphate when working over a sufficient interval of E'_h. Such a system need not involve an intermediate metabolite nor need the oxido-reduction involve the transfer of hydrogen. Thus coenzymes I and II flavoproteins or cytochromes could act as phosphorylating carriers, if a mechanism existed for the transfer of phosphate.

A necessary but not sufficient proof that a given substance can act as a phosphorylating carrier is that its oxidation can be shown to lead to phosphorylation. Such evidence is entirely lacking except for the carrier system postulated by Szt.

Györgyi (73), consisting of the succinate-fumarate or malate-oxaloacetate pairs; phosphorylation has been shown to accompany the oxidation, as substrates, of both malate (25) and succinate (12, 13), and it is therefore possible that it might accompany their oxidation as carriers.

Succinate and Malate as Carriers. It may be said at the outset that the evidence for the participation of succinate and malate in hydrogen transport is far from conclusive, but there is perhaps too much favorable evidence for the Szt. Györgyi scheme to be completely neglected. There is no experimental evidence that the dicarboxylic acids lead to phosphorylation when acting as carriers, but no one appears to have looked carefully for this or for any effect which their presence might have on the P:O ratio. (The system in which this should be most clearly detectable, if it occurs, is that in which Szt. Györgyi first claimed to have shown their activity in the transport of hydrogen, namely in the oxidation of glycogen, glucose or an intermediate to pyruvate.)

We shall therefore reexamine briefly the evidence that succinate or malate can be active in hydrogen transport. Szt. Györgyi's original claim was based on the activity of the enzymes malic dehydrogenase, fumarase and succinic dehydrogenase, the ability of the last to react with cytochrome C and the now well-known effects of the dicarboxylic acids in increasing and stabilizing the rate of oxidation of glucose by tissues which have been ground, dialyzed or otherwise damaged. These facts are not in doubt and have been widely confirmed by later workers. The probability that the succinate-fumarate reaction is significant in the transfer of hydrogen from substrates which reduce CoI was lessened by the failure to demonstrate a rapid reaction of fumarate with H_2CoI (16) and by the independence of succinic dehydrogenase of CoI. Furthermore, Potter (63) demonstrated that a reconstructed enzyme system could oxidize triose phosphate rapidly in the presence of enough malonate to prevent completely the oxidation of added succinate. Straub (68) has also shown that washed muscle does not need succinate to dehydrogenate malate. It seems to be agreed that succinate is not a carrier; but, as we have seen, a difference of enzymatic specificity is needed anyway if a hydrogen carrier is also to phosphorylate, and it is not necessary to assume that a carrier forms an essential link. The question of the participation of succinate in hydrogen and phosphate transport should not therefore be regarded as quite closed.

Other work bears on the activity of the malate-oxaloacetate system. (Although it is often fumarate that is added, the presence of fumarase makes this equivalent to malate.) Several workers have shown that, on the addition of these acids, true catalytic effects occur, that is, an increase of oxygen uptake which cannot be accounted for by the oxidation of the added dicarboxylic acid as a substrate. Thus Stare (65) and Stare and Baumann (66) showed clearly the catalytic effect of small quantities of fumarate in muscle preparations, this increasing with the damage to the tissue. Citrate had much smaller effects. Colowick *et al.* (13) showed that succinate is catalytic in the oxidation of glucose and pyruvate by homogenized kidney; Banga *et al.* (3) showed that citrate and α-ketoglutarate are much less active than are the dicarboxylic acids in dispersions of brain. The interpretation of these catalytic effects is made doubtful, however, by the existence of an alternative

explanation, namely, that oxaloacetate acts by condensing with pyruvate or a product of pyruvate in the 'modified citric acid cycle' (31). None of the experiments quoted can distinguish between these two explanations. In either case, it is oxaloacetate which is believed to cause the catalytic increase in respiration, either by acting as a hydrogen carrier or by condensing with 'pyruvate,' and other intermediates are catalytic only insofar as they give rise to oxaloacetate. Krebs' (31) demonstration that various members of the cycle are equivalent as substrates (at much above the concentrations needed for a catalytic effect) does not affect this conclusion, because of the possibility of saturation of an enzyme mediating a later stage.

Another line of evidence is the demonstration that oxaloacetate can accept hydrogen from various substrates, although this evidence is permissive rather than cogent. Szt. Györgyi (72) and Parnas and Szankowski (62) showed that during 'carbohydrate' oxidation the reduction of oxaloacetate to malate occurs. (Negelein and Brömel, 53, 54, have shown that this oxidative stage involves phosphoglyceraldehyde.) Breusch (10) demonstrated the anaerobic reduction of oxaloacetate, and Krebs *et al.* (30–34) showed that oxaloacetate is reduced to malate by hydrogen from pyruvate plus oxaloacetate and by citrate. However, the demonstration of the anaerobic reduction of oxaloacetate does not prove that this is of importance under aerobic conditions and again another explanation of the aerobic formation of malate from oxaloacetate is possible. Thus, the 'modified citric acid cycle' allows malate to be generated aerobically through an oxidative process, so that the demonstration of its formation does not prove that it was formed reductively from oxaloacetate. (This possibility does not exist in the anaerobic experiments.) We conclude that, on the experimental evidence, the case for succinate or malate as hydrogen carriers is neither proved nor disproved.

One general objection to the idea of the malate-oxaloacetate system will be discussed further. This has been forcibly stated by Ball (1), that since, as far as is known, oxaloacetate must receive hydrogen from and malate give up hydrogen to CoI, "we are right where we started" and the scheme seems "pointless".

Diffusion in Hydrogen Transport. Unless all the enzymes concerned with the oxidation of a substrate are arranged in the cell with their active groups closely adjacent, diffusion through space of carrier molecules must occur. What evidence there is suggests that they are not so arranged. The proteins concerned (dehydrogenases, flavoproteins, cytochromes) could diffuse relatively slowly, if at all, and transport from one to another must be performed by smaller molecules. We may suppose that the coenzymes perform such a function. The rate of oxidation might then depend on the rate of their diffusion as well as upon the rates of their reactions with substrate (at the surface of a dehydrogenase molecule) and with flavoprotein. In order to investigate this possibility theoretically, we choose the case where the molecules of protein occur randomly in the solution. A molecule of coenzyme I or II is considered to react immediately on collision with an enzyme molecule so that the rate of diffusion determines the rate of hydrogen transport.

Let the *number of molecules* per ml. of dehydrogenase be n_1 and of flavoprotein n_2; let the *concentrations* of unbound Co be c_1 and of unbound H_2Co be c_2 moles per liter; let the effective collision areas of Co with dehydrogenase and of H_2Co with

flavoprotein be A_1 and A_2 cm.^{2}, respectively, and the diffusion constant of Co and H_2Co be D cm.^{2} sec.$^{-1}$

Consider the diffusion of a molecule of H_2Co, which has just been hydrogenated. Irrespective of the direction of its diffusion, the probability that its first effective collision with a molecule of flavoprotein will occur at a distance between x and $x + dx$ from its position at zero time is $n_2A_2 \cdot e^{-n^2A^2x}\,dx$. According to Einstein (17) the time taken for a particle to diffuse a distance x is $t = x^2/2D$. The *mean time* taken by all molecules of H_2Co, from the moment of hydrogenation to that of the next dehydrogenation, is therefore:

$$\bar{t}_2 = \frac{1}{2D}\int_0^\infty n_2 A_2 \cdot e^{-n_2A_2x} \cdot x^2 \cdot dx \Big/ \int_0^\infty n_2 A_2 \cdot e^{-n_2A_2x} \cdot dx = 1/Dn_2^2A_2^2$$

Similarly the mean time for the reverse process is $\bar{t}_1 = 1/Dn_1^2A_1^2$; for a steady state, $c_1\bar{t}_2 = c_2\bar{t}_1$; and the rate of hydrogen transport is given by $c_1/\bar{t}_1 = c_2/\bar{t}_2$ moles/liter/second.

This result is of no interest unless it can be given a quantitative value, and in order to do this, we shall have to guess fairly boldly. Schlenk (64) indicates that the total concentration of all dehydrogenases is 1 to 2 grams per 100 ml. in muscle, and, if an average molecular weight of 35,000 is assumed, this makes n_1 about $N \times 4 \times 10^{-7}$ molecules/ml. (where N is the Avogadro number 6.06×10^{23}). Ball (1) gives a value for the concentration of flavoproteins of 8×10^{-5} molar, whence n_2 is about $N \times 8 \times 10^{-8}$ molecules/ml. He also gives 25×10^{-5} molar for the total concentration of CoI.

However, a fraction of the Co is bound by dehydrogenases and by flavoproteins; the values of K_m for the combination of CoI with dehydrogenases seem to lie between 10^{-4} and 10^{-5} molar (e.g., Negelein and Wulff, 55; Green, 19; Green and Brosteaux, 20; Needham *et al.*, 52), and Haas (21) gives 10^{-5} for the K_m of combination of H_2CoII with cytochrome reductase. Thus, taking a mean value for all K_ms of 3×10^{-5}, the concentration of *free* coenzymes ($c_1 + c_2$) is about 3×10^{-5} molar.

The radius of a protein molecule is about 2×10^{-7} cm. so that its spherical area is 5×10^{-13} cm.^{2} However, the *effective* collision area is likely to be much less than this, since only a part of the enzyme surface will be active and since the Co and enzyme probably must collide within a certain range of relative orientation for reaction to occur. We assume, therefore, an effective collision area 50 times less, that is of 10^{-14} cm.^{2} (= 100 Å^2). D is likely to be about 5×10^{-6} cm.^{2} sec.$^{-1}$

Using these values

$$\bar{t}_1 = 0.034 \text{ seconds and } \bar{t}_2 = 0.85 \text{ seconds}$$
$$c_1 = 0.11 \times 10^{-5} \text{ molar and } c_2 = 2.9 \times 10^{-5} \text{ molar.}$$

Thus the rate of transport of hydrogen by diffusion becomes 3.3×10^{-5} moles/liter/second. This value may be compared with observed values of the $Q(O_2)$ of whole muscle, a reasonable value for which is -10. Such a $Q(O_2)$ corresponds to about 10^{-4} moles/liter/second for the whole volume of the muscle.

This result indicates that the diffusion of coenzyme could be a limiting factor in the transport of hydrogen and thence in the total process of oxidation. It would

be easy, by modifying the quantitative assumptions, to obtain much higher or much lower predicted values for the rate of the diffusion process, but we regard the assumptions which we have made as reasonable. Moreover, arrangements in space of the proteins other than random would also make a great difference to the demands on diffusion. If the dehydrogenases were closely grouped with the flavoproteins the demand would be less, but if the flavoproteins and dehydrogenases were separately grouped, at a distance from each other, the importance of diffusion would be still greater.

One can now see what might be the importance of the dicarboxylic acids in hydrogen transport; they could relieve the diffusion 'bottleneck' by acting as more rapid carriers between the two enzyme systems—oxaloacetate receiving hydrogen from H_2CoI at one site and the malate, after diffusion, passing hydrogen to CoI and thence to flavoprotein at another site. This facilitating effect could be considerable because of the relatively high concentration of unbound dicarboxylic acids, though their presence would not be essential to the transport of hydrogen. At the same time, energy could become available for phosphorylation, provided that the conditions discussed earlier were fulfilled.

It is to be noted that this scheme implies the close spatial apposition of the enzyme which dehydrogenates malate or phosphomalate (whichever is concerned) to the flavoprotein and of that which hydrogenates oxaloacetate to the substrate dehydrogenases.

V. KINETIC PROBLEMS

We have seen that the amount of energy available from the oxidative process is dependent on the E'_h at which oxidation of the substrate effectively takes place. There is no evidence that phosphorylation accompanies the oxidation of any substances other than intermediary metabolites, and Ochoa (57) found no phosphorylation during the oxidation of H_2CoI. If we assume, in accordance with this evidence, that all oxidative phosphorylation is associated with the oxidation of metabolites by CoI or CoII, then the E'_h at which the coenzymes oxidize the substrate determines the energy available and therefore limits the amount of phosphorylation. Table 5 shows that E'_h must be at least +0.26 volt to give a P:O ratio of 1.8 in the oxidation of pyruvate, whether a carrier is involved or not.

Since the E'_0 of uncombined CoI is −0.28 volt (8), at E'_h+0.26 volt the ratio of uncombined $[H_2Co]/[Co]$ is 10^{-18}. The total concentration of uncombined Co is of the order of 10^{-4} molar, so that the concentration of uncombined H_2Co is 10^{-22} molar. (This conclusion holds whether it is considered that dehydrogenation of the substrate takes place directly or that the malate-oxaloacetate system intervenes.) At such a concentration the rate of reaction would be effectively zero since *a*) the rate of collision between H_2Co and flavoprotein is effectively zero; and *b*) the rate of reaction of the H_2Co-flavoprotein complex, in equilibrium with 10^{-22} molar uncombined H_2Co, would be effectively zero. (Assuming that Hogness' (23) values for K_m and K_r, for the complex between H_2CoII and cytochrome reductase, apply here, the concentration of this complex would be 10^{-21} molar and its rate of reaction 3×10^{-20} moles/liter/second.)

Clearly someting is wrong. In order to account for the observed phosphorylation and an adequate rate of oxidation, we must assume one or both of the following:

1 *a*) that the rate of collision between *uncombined* H_2Co and flavoprotein does not limit the rate, because hydrogen is transferred from the substrate or a carrier, directly to Co which is already in combination with flavoprotein. This necessarily implies that the dehydrogenase of the substrate or carrier forms a part of a complex with flavoprotein and *b*) that there is a very much larger concentration of the H_2Co-flavoprotein complex in equilibrium with 10^{-22} molar uncombined H_2Co than Hogness' figures would suggest, this being necessary in order to give an adequate rate of reaction of the complex. This would mean that K_m must have a very low value, of the order of 10^{-20}, compared with the value 10^{-5} found by Hogness.

2) that the initial assumption is wrong, though it was suggested by the experimental data, and that further phosphorylation can accompany the oxidation of some carrier system or systems of E'_0 higher than that of the coenzymes, such as succinate, flavoprotein or cytochrome. No mechanism of phosphate transfer by the last two has been proposed.

Even with *assumption 2*), it is necessary that the E'_h at which coenzyme is oxidized should be, at the lowest, -0.02 volt if phosphorylation of creatine is to accompany the oxidation of pyruvate intermediates by coenzyme. This would imply a concentration of about 10^{-13} molar for uncombined H_2Co, which is still very low. Furthermore, there is some difficulty in understanding the kinetics of any carrier system which is to produce 16,700 calories per mole of hydrogen transferred since it must work over an interval of E'_h of at least 0.36 volt. Optimally, its E'_0 would lie in the middle of this interval, when its upper and lower values of E'_h would then correspond to ratios of oxidized to reduced forms of 10^6 and 10^{-6}.

More experimental evidence bearing on these problems is needed for further discussion of them to be profitable.

VI. CONCLUSION

We have surveyed the evidence which bears on the relationship between oxidation and phosphorylation mainly from the viewpoints of thermodynamics and kinetics. The result has been to set limits and to emphasize the existence of problems rather than to make a positive contribution towards the understanding of these processes, but we hope that our criticism may be a help to future work. In particular:

1) We doubt whether the present evidence justifies the assumption of a P:O ratio greater than 2 in the oxidation of pyruvate.

2) According to our present knowledge it is difficult to understand on kinetic grounds how a P:O ratio even as low as 2 can be achieved at an appreciable rate of reaction. Light should be thrown on this problem as information accumulates about the spatial distribution of enzyme systems in cells and about the possibility of phosphorylation accompanying any oxidative processes other than those of substrates by pyridine nucleotide coenzymes.

3) It has been shown that catalytic effects of intermediates on oxidation might be due to their assisting in the diffusional transport of hydrogen. In this connection, the question of the spatial distribution of enzymes is again of importance. Such an

action of intermediates could be combined with that of phosphate transport. Information on these two aspects might be best obtained from the study of some simple oxidative process, such as that of phosphoglyceraldehyde to phosphoglyceric acid.

In general, we have tried to indicate how chemical, thermodynamic and kinetic types of evidence may supplement each other in leading to the understanding of cellular processes.

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REFERENCES

1. BAILEY. *Proc. Biochem. Society*, April 9, 1948.
1a. BALL. *Symposium on Respiratory Enzymes*. Wisconsin: 1942. Pp. 16, 35.
2. BANGA. Quoted on p. 81 of Szt. Györgyi, *Acta physiol. Scand.* 9, Suppl. XXV, 1945.
3. BANGA, OCHOA AND PETERS. *Biochem. J.* 33: 1109, 1939.
4. BARRON. *Physiol. Rev.* 19: 184, 1939.
5. BARRON. *Adv. Enzymology* 3: 149, 1943.
6. BARRON AND HASTINGS. *J. Biol. Chem.* 107: 567, 1934.
7. BELITZER AND TZIBAKOWA. *Biokhemiya* 4: 516, 1939.
8. BORSOOK. *J. Biol. Chem.* 133: 629, 1940.
9. BORSOOK AND SCHOTT. *J. Biol. Chem.* 92: 535, 1931.
10. BREUSCH. *Biochem. J.* 33: 1757, 1939.
11. BÜCHER. *Naturwissenschaften* 30: 756, 1942.
12. COLOWICK, WELCH AND CORI. *J. Biol. Chem.* 133: 359, 1940.
13. COLOWICK, KALCKAR AND CORI. *J. Biol. Chem.* 137: 343, 1941.
14. COLOWICK AND KALCKAR. *J. Biol. Chem.* 148: 117, 1943.
15. CORI, CORI AND GREEN. *J. Biol. Chem.* 151: 39, 1943.
16. DEWAN AND GREEN. *Biochem. J.* 31: 1074, 1937.
17. EINSTEIN. *Ann. Physik.* 19: 371, 1906.
18. GERARD AND TUPIKOWA. *J. cell. comp. Physiol.* 12: 325, 1938.
19. GREEN. *Biochem. J.* 30: 2095, 1936.
20. GREEN AND BROSTEAUX. *Biochem. J.* 30: 1489, 1936.
21. HAAS. *Symposium on Respiratory Enzymes*. Wisconsin: 1942. P. 43.
22. HERBERT, GORDON, SABRAHMANYAN AND GREEN. *Biochem. J.* 34: 1108, 1940
23. HOGNESS. *Symposium on Respiratory Enzymes*. Wisconsin: 1942. P. 134.
24. JOHNSON. *Biochem. J.* 33: 1046, 1939.
25. KALCKAR. *Biochem. J.* 33: 631, 1939.
26. KALCKAR. *Chem. Rev.* 28: 71, 1941.
27. KALCKAR. *Biol. Rev.* 17: 28, 1942.
28. KALCKAR. *J. Biol. Chem.* 148: 127, 1943.
29. KHARASCH. *J. Res. Nat. Bur. Standards* 2: 359, 1929.
30. KREBS. *Biochem. J.* 34: 775, 1940.
31. KREBS. *Adv. Enzymology* 3: 191, 1943.
32. KREBS AND JOHNSON. *Enzymologia* 4: 148, 1937.
33. KREBS, SMYTH AND EVANS. *Biochem. J.* 34: 1041, 1940.
34. KREBS, EGGLESTON, KLEINZELLER AND SMYTH. *Biochem. J.* 34: 1234, 1940.
35. LAKI. *Z. physiol. Chem.* 249: 63, 1937.
36. LARDY AND ZIEGLER. *J. Biol. Chem.* 159: 343, 1945.
37. LEHMANN. *Skand. Arch. Physiol.* 58: 173, 1929.
38. LEHMANN. *Biochem. Z.* 281: 271, 1935.
39. LEHNARTZ. *Z. physiol. Chem.* 184: 1, 1929.
40. LIPMANN. *Adv. Enzymology* 1: 99, 1941.
41. LIPMANN. *Ann. Rev. Biochem.* 12: 1, 1943.

42. LIPMANN. *Adv. Enzymology* 6: 231, 1946.
43. LOHMANN. *Biochem. Z.* 203: 172, 1928.
44. LOHMANN AND MEYERHOF. *Biochem. Z.* 273: 60, 1934.
45. LUNDSGAARD. *Biochem. Z.* 233: 322, 1931.
46. MEYERHOF. *Symposium on Respiratory Enzymes.* Wisconsin: 1942. P. 3.
47. MEYERHOF AND JUNOWICZ-KOCHOLATY. *J. Biol. Chem.* 149: 71, 1943.
48. MEYERHOF AND LOHMANN. *Biochem. Z.* 196: 22, 1928.
49. MEYERHOF, OHLMEYER AND MÖHLE. *Biochem. Z.* 297: 113, 1938.
50. MEYERHOF AND SCHULZ. *Biochem. Z.* 297: 60, 1938.
51. NEEDHAM AND PILLAI. *Biochem. J.* 31: 1837, 1937.
52. NEEDHAM, GREEN AND DEWAN. *Biochem. J.* 31: 2327, 1937.
53. NEGELEIN AND BRÖMEL. *Biochem. Z.* 301: 135, 1939.
54. NEGELEIN AND BRÖMEL. *Biochem. Z.* 303: 132, 1939.
55. NEGELEIN AND WULFF. *Biochem. Z.* 293: 351, 1937.
56. OCHOA. *J. Biol. Chem.* 138: 751, 1941.
57. OCHOA. *J. Biol. Chem.* 151: 493, 1943.
58. OCHOA. *J. Biol. Chem.* 155: 87, 1944.
59. OCHOA. *J. Biol. Chem.* 159: 243, 1945.
60. OCHOA AND WEISZ-TABORI. *J. Biol. Chem.* 159: 245, 1945.
61. PARKS AND HUFFMAN. *Free Energies of Organic Compounds.* New York: 1932.
62. PARNAS AND SZANKOWSKI. *Enzymologia* 3: 220, 1937.
63. POTTER. *J. Biol. Chem.* 134: 417, 1940.
64. SCHLENK. *Adv. Enzymology* 5: 207, 1945.
65. STARE. *Biochem. J.* 30: 2257, 1936.
66. STARE AND BAUMANN. *Proc. Roy. Soc. Lond. B* 121: 338, 1936.
67. STOTZ, SIDWELL AND HOGNESS. *J. Biol. Chem.* 124: 11, 1938.
68. STRAUB. *Enzymologia* 9: 148, 1940.
69. SUTHERLAND, COLOWICK AND CORI. *J. Biol. Chem.* 140: 309, 1941.
70. GÖZSY AND SZT. GYÖRGYI. *Z. physiol. Chem.* 224: 1, 1934.
71. ANNAU, BANGA, GÖZSY, HUSZAK, LAKI, STRAUB AND SZT. GYÖRGYI. *Z. physiol. Chem.* 236: 1, 1935.
72. ANNAU, BANGA, BLASZO, BRUCKNER, LAKI, STRAUB AND SZT. GYÖRGYI. *Z. physiol. Chem.* 244: 105, 1936.
73. SZT. GYÖRGYI. *Studies on Biological Oxidation.* Budapest. 1937.
74. WARBURG AND CHRISTIAN. *Biochem. Z.* 303: 40, 1939.
75. WENNER. *Thermochemical Calculations.* New York: 1941.

ALLOXAN DIABETES

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EXPERIMENTAL DIABETES was first produced by total or partial pancreatectomy. The second method of inducing diabetes was by means of anterior pituitary extract. The third form of experimental diabetes was produced in 1943 by the administration of alloxan after the discovery by Dunn, Sheehan and McLetchie (59) that this substance caused necrosis of the pancreatic islets. These persistent or permanent forms of experimental diabetes are to be distinguished from the various temporary glycosurias or hyperglycemias. The literature on alloxan diabetes will be reviewed under the following headings: I. History and general description of alloxan diabetes; II. The mechanism of action of alloxan on susceptible cells, especially those of the islands.

HISTORY, NATURE AND COURSE OF ALLOXAN DIABETES

Early Bio-Chemistry of Alloxan. Alloxan was first made by Woehler (172) in 1838 by the oxidation of uric acid. Since then there have been only a few references to its biological significance. In 1862 Liebig (133) found alloxan in the intestinal mucus of a patient and in 1866 Lang (124) reported a patient who excreted alloxan or a precursor thereof in the urine. The tests were probably valid by modern appraisal (3). Early studies (4, 149) on the reversible reaction, uric acid $\rightleftarrows$ dialuric acid, indicate that alloxan may be present in traces in blood (3). The alloxan-dialuric acid oxidation-reduction system has been described (150, 171). The reactions of alloxan with sulfur compounds (132) and its action as a capillary poison have been studied (122). Its excretion was investigated by Cerecedo (34) and little has been added by later investigators. Cerecedo (34) cites older references indicating that alloxan is to a large extent destroyed in the body and describing the red color of the urine after its ingestion in dogs and mice. Although a fraction of the alloxan may be converted into alloxantin and excreted in the urine as murexide most of it was thought to be eliminated in the bile, perhaps as ethereal sulfate. There was a slight increase in the total nitrogen output but the urea figures showed that alloxan was not broken down to urea. In every case there was a drop in the inorganic sulfates on the day after its administration which was by ingestion with food. The fall in inorganic sulfates was not compensated by a rise in any other sulfur fraction of the urine, hence the assumption that it was possibly excreted by the bile. He also concluded that alloxan was not a physiological breakdown product of uric acid. Dilute concentrations of alloxan increased the oxygen uptake of liver suspensions and greatly accelerated their oxidation of ethyl alcohol (18). Alloxan inhibited the conversion of the Cori ester to the Robison ester (129). The acute hypoglycemic action of alloxan was first described by Jacobs (104).

Determination of Alloxan. Since the discovery of alloxan diabetes numerous methods for the quantitative determination of alloxan have been developed. The first to appear was that of Leech and Bailey (128) which, though of limited specificity, demonstrated the instability of alloxan in blood, a finding which has been fully confirmed (3, 22, 84, 115). In his article describing six methods for the determination of alloxan, Archibald (3) gives a detailed review of the chemistry of alloxan and of the problems of specificity, accuracy and choice of these methods. The fluorometric methods (3, 115) which are the most sensitive have shown that if alloxan is present in normal blood there is less than 10–20 gamma per 100 cc. These results appear to disagree with the reports (152, 167) that, by qualitative tests, alloxan or its reduction products may be a normal constituent of certain tissues. A microbiological and another fluorometric method (15) as well as another colorimetric method applied to blood and tissues (22) provide the investigator with a large choice of procedures. In addition to other chemical considerations the extent to which related compounds may be converted *in vivo* to alloxan or to its reactive derivatives must be taken into account in the interpretation of results obtained by these procedures.

Production and Course of Alloxan Diabetes. This has been previously reviewed (51, 73, 77, 112, 113). The background of the original observation in rabbits is cited in the authors' words (59).

> "Experiments were being conducted recently by one of us (J. S. D.) with the object of elucidating the pathogenesis of the renal lesion of the crush syndrome and of the somewhat similar condition which has been observed to follow the accident of a mismatched blood transfusion. In the course of these experiments trial was made by intravenous injection of a number of substances which might influence particularly the lower segments of the renal nephrons. Among other lines investigated in this way uric acid and related products were exhibited, as it had been found by Dunn and Polson (1926) that uric acid, suitably applied experimentally, could produce a selective lesion in the renal second convoluted tubules and collecting tubules. Among the substances in this series alloxan was found initially to give very promising results which may be recorded at another time. A difficulty which arose, however, in the successful establishment of a tubular nephritis by use of alloxan was that with the order of dosage required for this purpose many animals died during the first day or so with distinctive symptoms which were not referable to renal disease. It was in the investigations of these early deaths that a lesion was discovered in the islets of Langerhans."

These authors found the initial rise and subsequent fall in blood sugar, and they proposed the search for animals that would survive with diabetes. This was soon achieved by them (58) and by others (5, 74).

Species. Following the original announcement that alloxan caused necrosis of the islets, permanent diabetes was soon produced in rabbits (5, 39, 52, 54, 117, 118), rats (53, 58, 80, 86, 102, 116, 126) and dogs (33, 74, 93, 111, 159, 166). In the successful experiments, diabetes was apparent in 24 hours and was permanent. In certain species with few beta cells, such as the sheep, pigeon and frog, it has been difficult to define islet lesions. Although minor lesions of the islands are reported in the toad, diabetes does not occur, in contrast to the response of this species to pancreatectomy. Before discussing the general range of dosage given in table 1, certain general comments may be made. The use of alloxan has enlarged our knowledge of diabetes in different species because alloxan diabetes can be produced in those animals with a diffuse pancreas. The ease with which the rat, in particular, is made available

for the study of diabetes is a boon to investigators. In general the use of alloxan has confirmed previous knowledge of the response of different animals to diabetes. Thus, the duck does not have the sustained hyperglycemia after pancreatectomy or after alloxan. Any type of diabetes in ruminants is relatively mild; in carnivora, it is usually severe. Like the toad, the carnivorous birds (owls) do not develop diabetes after alloxan, presumably because the beta cells are resistant to the drug. When a new species is used three variables must be kept in mind: species difference in response to the lack of insulin; the variable susceptibility of different animals to alloxan; and the variable severity of the diabetes produced by alloxan in susceptible animals. The uratic deposits in birds will be discussed under complications.

TABLE 1. STUDIES ON ALLOXAN IN VARIOUS SPECIES

SPECIES	LESIONS OF ISLETS	HYPERGLYCEMIA (DIABETES)	DOSAGE	URATIC DEPOSITS	REFERENCES AND NOTES
			mgm/kgm.		
Rabbit	+	+	25–300		see text
Rat	+	+	100–200		see text
Hamster	+	+	40–200		(87)
Dog	+	+	50–200		see text
Cat	+	+	150		(148, 154)
Sheep	+	+	90		(60, 109, 110)
Monkey	+	+	300		(12)
Man	+	+	100–600		see later section
Frog	+	o	50–1000		(158)
Toad	o	o	200		(21, 96)
Turtle	–	+	400		(69)
Pigeon	+	+	75–200	+	(78, 157)
Duck	+	o	50–750	o	(143, 157)
	o	o	200–300	o	(157)
Chicken	o	o	75–400	+	(143, 157)
Barn owls	o	o	50–200	+	(157)
Horned owls	o	o	100–250	o	(157)

+ = present; o = absent; – = no data.

Dosage, Toxicity and Methods of Administration. Table 1 surveys the range of dosage used. The susceptibility to both toxic and diabetogenic doses varies widely not only in different species but also among animals of the same species. In the common laboratory animals (rat, rabbit, dog) the picture described by Goldner and Gomori (74) has been confirmed. They defined the response of the dog to single intravenous doses of alloxan as follows:

125–200 mgm/kgm.: Sudden death with convulsions.
75–100 mgm/kgm.: Diabetic-uremic syndrome.
50–75 mgm/kgm.: Diabetes
25 mgm/kgm.: No observed effect.

Thus for each species the lethal, the nephropathic and the diabetogenic doses must be considered.

The symptoms and findings after toxic doses of alloxan were first described by Jacobs (104) and by Dunn *et al.* (57–59). The latter noted early death from hypo-

glycemia or from uremia of varying severity at one- to five-day intervals. In the light of subsequent work the following events may occur after a single intravenous dose. *a*) Death may occur in a few hours in convulsions without hypoglycemia or nitrogen retention and without anatomical lesions. This is unexplained in the dog (33, 74) and has been attributed to toxic action on the nervous system in toads (21, 96), although no lesions of the nervous system have been reported. *b*) Acute pulmonary edema has been seen in dogs (93) and cats (148). The resultant anoxia might be a cause of convulsions. *c*) Death may occur at 6–18 hours from hypoglycemia. *d*) Death often occurs in one to five days from uremia with oliguria, albuminuria, anuria or hematuria, usually associated with diabetes (45, 57, 58, 59, 74, 82). Table 2 gives the data on the minimum lethal dose. The toxicity is greatest after intravenous and least after subcutaneous administration. The coincidental agreement between the LD_{50} (95) and ED_{50}, the minimum effective or diabetogenic dose (25), does not alter the practical ease of preparing diabetic rats. The subcu-

TABLE 2. MINIMUM LETHAL DOSE (LD_{50}) OF ALLOXAN

ANIMAL	ROUTE OF ADMINISTRATION	LD_{50}	REFERENCES	NOTES
		mgm/kgm.		
Rat	intravenous	49	(95)	
	intravenous	45[a]	(25)	(a) ED_{50}—see text
	intraperitoneal	152	(95)	
	intraperitoneal	360–140	(72)	5 to 200 grams body weight
	subcutaneous	175[b]	(116)	(b) 90% diabetogenic; see text
Hamster	intravenous	65.4 ± 9.8[c]	(87)	(c) S.E.
Pigeon	intravenous	118.8 ± 11.8	(157)	
Rooster	intravenous	199.8 ± 19.4	(157)	

taneous dose of 175 mgm. per kgm. (116) is not a calculated LD_{50} but is included because of its usefulness.

The production of diabetes without nephritis or other demonstrable injury is dependent on a practical difference between the nephrotoxic and diabetogenic doses. In rabbits and dogs this difference is such that a fair proportion of animals will have uncomplicated diabetes if a suitable dose is used. The margin is narrower in rats, for diabetes could not be obtained without an elevation of blood non-protein nitrogen (16). However, as the islet injury is usually permanent and the renal damage is usually reversible, an essentially uncomplicated diabetes may be secured. A few examples should be cited of the irregularity with which diabetes is produced. "Of 43 dogs injected only 7 developed a high degree of glycosuria without serious complications" (166). In 30 dogs given 75 mgm. per kgm. by Houssay *et al.* (93) only 8 survived more than a week with diabetes; with the dose of 50 mgm. per kgm., 20 of 70 dogs were permanently diabetic. Data on other doses are given (93). In rabbits 10 out of 13 (54), 12 out of 27 (118), and 53 out of 56 (52) were made diabetic, all on

the dose of 200 mgm. per kgm. intravenously. The response of rabbits to various doses has been described (86). Subsequent knowledge of the influence of diet and other factors accounts in part for the variations of different workers (see below).

Alloxan is given in freshly prepared solutions of 1 to 5 per cent concentration in water or saline. From what has been said intravenous administration is the route of choice in most animals. The subcutaneous or intraperitoneal route is quite satisfactory in rats although in rabbits doses up to 700 mgm. per kgm. intraperitoneally were entirely ineffective (118). Diabetes has been produced in rats (153) and rabbits (155) by the injection of alloxan into the alimentary canal and in cats (154) if alloxan was rapidly ingested with food by fasted animals. However, oral administration is usually unsatisfactory.

The repetition of small doses of alloxan which are ineffective singly may lead to diabetes. In rabbits, doses of 40 and 50 mgm. per kgm. daily for 4 to 13 days have produced permanent diabetes (6, 118). Nephritis is largely avoided by this procedure. More accurate studies in the rat have shown a progressive diminution in glucose tolerance on as little as 25 mgm. per kgm. per day intravenously for 4 weeks (161). Dogs likewise respond to repeated small doses (93). Thus, a minimal effective concentration of alloxan causes progressive damage to the islands. This explanation is chosen because the injury is histologically apparent after small doses (56) and because the rapid disappearance of alloxan from the blood (3, 22, 115, 128) makes a cumulative action unlikely.

Diet and Other Conditions Influencing the Response to Alloxan. Martinez (136) found that rats were more sensitive to a constant dose of alloxan after a foreperiod on a high fat diet (90–100 per cent mortality) and less sensitive after a high carbohydrate and protein diet (33–40 per cent mortality). In their paper on the production of alloxan diabetes, Kass and Wasibren (116) studied the influence of fasting on the response to alloxan. Withdrawal of all food from adult rats for 48 to 60 hours rendered them almost uniformly susceptible to the subsequent administration of 175 mgm. per kgm. subcutaneously. Ninety to 95 per cent became diabetic where as only 25 per cent of animals which had not been fasted responded to a similar dose. Older and heavier rats required a longer period of starvation. Several authors have observed that animals which have not responded to one injection may be refractory to the reinjection of a similar dose of alloxan (74, 86, 93, 116, 118). These results have been partly explained by Kass and Wasibren (116) who found that in their animals this occurred in the group which had not been fasted. After fasting for 60 hours the refractory animals became diabetic on the standard dose. The value of these observations is apparent. When the intravenous route is used, the rate of injection is important (93), for the slow injection of an otherwise effective dose produced no diabetes. Rats of both sexes were equally sensitive but wide differences in response were associated with variations in body weight (23, 72). In the detailed analysis of Gitter and Prieto Diaz (72 and table 2) the toxicity of alloxan changed in linear relation to the weight of the animal raised to the 0.73 power which suggested a direct relation between toxicity and this metabolic unit. They also applied this formula to an estimation of the LD_{50} for each rat. Barbiturate anesthesia renders animals more sensitive to the toxic effects of alloxan (118, 159), and this may also be true of chloralose

(98). If anesthesia is needed at the time alloxan is given, light ether anesthesia may be the safest (118). Other agents which enhance or prevent the action of alloxan will be discussed in relation to its mechanism of action.

Response of the Blood Sugar to Alloxan. Jacobs (104) first described the initial hyper- and hypo-glycemic phases following intravenous alloxan in rabbits, and observed the symptomatic relief of the hypoglycemia by the administration of glucose. Later, the phase of permanent hyperglycemia was added to the picture. Many reports have established the tri-phasic blood sugar curve associated with a diabetogenic dose of alloxan (5, 39, 54–58, 86, 104). This curve is composed of a rise in blood sugar at 2 to 4 hours after the administration of alloxan, a marked hypoglycemia at 6 to 12 hours, and a permanent hyperglycemia at 18 to 24 hours. The initial hyperglycemic phase does not occur in every case, whereas the hypoglycemic and diabetic phases are consistent except for minor variations in timing in different animals. Shipley and Beyer (159) observed a slight initial fall in blood glucose at 15 to 30 minutes and so refer to the curve as tetra-phasic. The knowledge that hypoglycemia caused early death in many instances led to the prophylactic administration of glucose (5, 54, 58). When animals were thus sustained during the period of hypoglycemia they survived and became permanently diabetic. Additional studies on the response of the blood sugar will be discussed under the mechanism of action of alloxan.

Duration and Constancy of the Diabetes. Alloxan diabetes may be permanent. This statement is true if one recognizes the experimental conditions to which it is applied and the exceptions that may be encountered. Diabetes has persisted for 5 to 11 months in rabbits (6, 52, 54, 118), 2 to 8 months in rats (28, 41, 58), 8 months in dogs (93) and 2 months in sheep (60, 109). As a rule the degree or severity of the diabetes remains constant but certain changes in its intensity have been seen. Thus, when less than the optimal dose was used, Houssay *et al.* (93) observed 3 dogs in which transitory diabetes lasted 5, 5 and 8 days. A similar example of spontaneous recovery in a dog after a few days of hyperglycemia had been previously reported (33). Slight but definite improvement in diabetes has followed the dietary treatment of diabetes in rats (28). Kass and Wasibren (116) state that 4 of 6 rats from the group which had not been starved before the injection of alloxan had normal blood sugars when tested three months after they had been shown to be hyperglycemic. Janes (104a) found that rats which had severe diabetes remained diabetic but that rats with mild initial diabetes tended to improve. When partial pancreatectomy is performed in young rats diabetes develops about three months later, i.e., after they have grown, and a similar increase in the manifestations of diabetes might well occur after alloxan under appropriate circumstances. Although this increase of severity with growth was not mentioned in the report on the dwarfing of young rats by diabetes (36), it has been suggested that diabetes may become more severe in the rat (27). A gradual increase in the severity of diabetes during the first three months has been noted in dogs (93) and in rabbits (5). In short, although alloxan diabetes may be permanent and constant, appropriate measurements of these characteristics must be made as required.

Severity of Diabetes. Alloxan diabetes should be regarded as partial pancrea-

tectomy produced by a chemical agent. This means that the subsequent diabetes may vary from the mildest to the most severe in type. There are many criteria for appraising the severity of diabetes, no one of which is entirely satisfactory for all situations. The glucose tolerance test reveals the earliest abnormalities in glucose metabolism but does not measure severity in a severely diabetic animal. Likewise the quantitative excretion of glucose and nitrogen during fasting, which are quite normal in mild diabetes, are valuable measurements of severe diabetes. Therefore the various measurements which have been made in alloxan diabetes must be evaluated with particular reference to the degree of 'chemical pancreatectomy' that has been achieved.

Hyperglycemia is an index of the presence of diabetes but is inadequate as a measure of its severity in the presence of glycosuria. In the dog, hyperglycemia has been of the degree usually seen in other types of experimental diabetes (e.g., 200–350 mgm. per 100 ml.). Rabbits and rats tend to have higher blood sugar levels (300–600 mgm. per 100 ml.) even when there is no apparent renal lesion and when the diabetes is mild as estimated by glycosuria (118, 127). Renal insufficiency may be responsible for some of the very high levels reported in alloxan diabetes. Diabetes with marked glycosuria and relatively lower blood sugar levels has been seen in sheep (60, 110). The glucose tolerance test varies from the slight impairment after small doses of alloxan to frankly diabetic curves (54, 65, 93, 97).

Diabetic animals respond readily to insulin and insulin resistance has not been encountered. Insulin tolerance or sensitivity tests resemble those seen in depancreatized animals (54, 74, 76, 93, 118). The diabetes may be controlled by the regular use of insulin, the dose of insulin varying with the severity of diabetes and the species used. The control of diabetes in rabbits is stated without details (54) and no data were found on the maintainance of diabetic rats, a species relatively resistant to insulin. In dogs, Houssay *et al.* (93) used 1 to 3 units per kgm. per day. In 3 dogs insulin treatment was followed by recovery but this cannot be distinguished from the spontaneous recoveries noted above. On the other hand 4 dogs treated for 25 to 45 days did not recover and this has been the experience of others with well established alloxan diabetes (166). The insulin requirement was comparable to that of pancreatic diabetes but they note (93) that the glycosuria of their animals was in general less than occurs after total pancreatectomy. Hence their results may not be comparable to those of Thorogood and Zimmerman (166) and Candela (29). In dogs with apparently severe alloxan diabetes the insulin requirement of fed animals was 3.3 to 3.8 units per kgm., calculated from the data of Thorogood and Zimmerman (166). After total pancreatectomy 1.1 to 1.4 units per kgm. were needed (166). Incidentally, the limitation of insulin requirement as a criterion of the severity of diabetes is shown in their experiments. Thus, a dog with severe glycosuria and moderate ketonuria from alloxan diabetes lived 91 days without insulin in contrast to 4 depancreatized dogs which died in coma 5–7 days after insulin was withdrawn. Although their insulin requirement was less, the depancreatized dogs had more severe diabetes by the criteria of survival, rate of loss of weight and acidosis. It would be interesting to know the insulin requirement during fasting in both types of diabetes. In any case, alloxan is a new aid in attacking such problems.

The quantitative excretion of glucose, nitrogen and acetone bodies has received little attention. In the dog, Houssay *et al.* (93) give values which in table 3 have been compared with their earlier figures for depancreatized dogs. One may assume that in alloxan diabetes as in pituitary diabetes occasional animals will be met which have 'total urinary diabetes', i.e., they are the equivalent of depancreatized animals in terms of these urinary metabolic measurements. In the dog the glycosuria and nitrogen excretion of pancreatic diabetes and phlorizin glycosuria are of the same order. Hence phlorizin glycosuria has been used as a basis for comparison in the case of the rabbit (118) in which there is little data on total pancreatectomy. If one uses an approximate estimate of the 'available glucose' of the diet (all the carbohydrate plus 50 per cent of the protein), the alloxan diabetic rats of Janes *et al.* (106) excreted about 80 per cent of the available glucose when fed. When fasted for one day the glycosuria was about 0.8 gram per kgm. per day, i.e., they had mild diabetes by comparison with the depancreatized dog. The proportion of the available glucose excreted by fed animals is a useful index of severity in moderately severe diabetes. Some such criteria are required if the degree of insulin deficiency is to be even approxi-

TABLE 3. EXCRETION OF GLUCOSE, NITROGEN AND ACETONE BODIES DURING FASTING[1]

DOGS	URINE				MAXIMUM WT. LOSS	SURVIVAL
	Glucose	Nitrogen	Ketones	D:N		
	g/k/d[2]	*g/k/d*	*mgm./k/d*		%	*days*
Depancreatized.....	2–4	0.7–1.8	—	2.8	50	5–30
Alloxan diab.....	1.2–2.8	0.5–1.3	10–50	2–2.7	35	90–240

[1] From Houssay *et al.* (91, 93).

[2] Grams per kilogram per day.

mated in alloxan diabetes. Ketonuria has been measured in fed dogs in which quantities up to 200 mgm. per day contrast with 1400 mgm. per day after pancreatectomy (166). In dogs and cats ketonuria usually appears when 80 per cent or more of the available glucose of the diet is excreted in the urine, so that it indicates relatively severe diabetes. Ketonuria has occurred in rabbits (5, 131) and rats (53, 58, 80) but its relation to quantitative glycosuria in these species is uncertain. The quantitative ketonuria of rats was increased by adding nicotinic acid to the diet (108). The mechanism of this is not understood. The description of alloxan diabetic rats as generally free from excessive ketone bodies but always on the borderline of ketosis, when they are severely diabetic, aptly summarizes the situation (108). Blood ketones have been elevated in some animals (74, 123) as would be expected from the presence of ketonuria.

Liver glycogen has been determined. Histologically it is diminished (80). On chemical determination, seven rabbits examined 3 hours to 25 days after alloxan had an average of 3.46 per cent of liver glycogen, a normal value (86). In diabetic rats (not fasted?) the average value of 0.83 per cent was obtained instead of 3.05 per cent in controls (123). In fasted diabetic rats, Weber (169) found liver glycogen higher than in the controls and a slightly higher level of glycogen in alloxan diabetes was

noted by Houssay *et al.* (97). Janes *et al.* (106) give an average of 0.88 per cent for diabetic rats in agreement with Weber (169). In diabetic acidosis the average liver glycogen was 0.06 per cent (114). In diabetic rats, muscle glycogen falls slightly (97, 123, 169) and cardiac glycogen increases strikingly in agreement with the results in other types of diabetes (123). The levels of liver and muscle glycogen were increased by the ingestion of glucose but the increment was much smaller in diabetic than in normal rats (97). The variation in the liver glycogen of the normal rat with different periods of fasting must be taken into account in reading these papers. The behavior of muscle and liver glycogen follows the pattern that would be expected in partial insulin deficiency of varying degree.

Complications. Death in acidosis and coma has been observed (6, 114). This occurs in a few days when large doses of alloxan cause the combination of severe diabetes and renal insufficiency. The carbon dioxide combining power of the blood was slightly decreased when dogs became drowsy although the NPN remained normal (74). In acute acidosis rats were semiconscious or comatose with hyperventilation, severe glycosuria and ketonuria (114). In this condition the changes in the blood and liver phosphate fractions resembled those recently described in diabetes mellitus.

The most striking complication of alloxan diabetes in rats and rabbits has been the development of cataract (6, 11, 36, 54, 131, 156, 160). This occurs as early as the sixth week in rats and at eight weeks in rabbits (131). The incidence of cataract is greater and the appearance time shorter when diabetes is severe, facts which explain the irregularity of this complication in different laboratories. Insulin treatment seemed to delay the development of cataract (11). Diabetic cataract resembles galactose cataract (144), cataract after partial pancreatectomy (40, 64) and the usual cataract in man (11). It is distinct from that produced by certain dietary deficiencies. In addition to cataract one group of investigators (131, 156) has reported the occurrence of retinal lesions, consisting of capillary hemorrhages seen by ophthalmoscopic and histologic examination. Exudates were not observed. Of their 21 diabetic rabbits 18 showed progressive hemorrhages. The retinal lesions were not related to the severity of diabetes and appeared as early as one month but usually three to four months after alloxan. No effect of insulin treatment is claimed. These investigators (131, 156) state that the plasma proteins were altered in all cases. Plasma albumin was decreased and beta globulin increased in severe diabetes and in all rabbits with hemorrhages. When plasma proteins were depleted by plasmapheresis before the production of diabetes retinal changes developed more rapidly.

Immature rats with alloxan diabetes were strikingly dwarfed, some remained sexually infantile and the intestinal tract was enlarged in proportion to body weight (36). The effect of alloxan diabetes on pregnancy and on the fetus has been observed in the dog (93, 142), rabbit (142) and rat (43, 44, 67, 142). In the 4 dogs reported there was one abnormal pregnancy, in another (93) the fetuses had normal blood sugars and the islands were normal 48 hours after birth. In rabbits there were many abortions, premature deliveries and stillbirths, only about half of the pregnancies going to term. There was no increased birth weight of the young rabbits. All of the pregnancies in diabetic rats were full term and the fetuses were of normal weight. In this connection, Miller (142) cites unpublished work of Long who observed that more severely

diabetic depancreatized rats had a high incidence of fetal reabsorption. Reabsorption of the fetus and its prevention by insulin treatment have been described by others (43, 44). In diabetic rats maternal hyperglycemia, fetal hyperglycemia and normal fetal blood sugar after delivery have been found (67). The fetal islands were normal despite the demonstration of alloxan in fetal blood. Bailey (113) found hyperplasia of the fetal islands but more information is needed on this point. In spite of poorly understood fluctuations in diabetes during pregnancy there is no definite or consistent relief of maternal diabetes of any type by the fetal islands. In contrast alloxan diabetes in the rat is alleviated by parabiotic union with a normal rat (160). The most interesting conclusion of all of these reports is that there may be differences in function between fetal and adult islands of Langerhans.

Lowry and Hegsted (134) showed that rats with alloxan diabetes had no increased tendency to develop thiamine deficiency, and that their thiamine requirement was less than that of normal controls. Neuritis has not been reported in alloxan diabetes.

The deposition of sodium urate on the serous surfaces in certain birds (table 1) is apparently a non-diabetic complication which makes it possible that alloxan may be useful in the experimental study of gout. Blood uric acid reached extremely high levels 24 hours after the injection of alloxan, and death occurred in 48 hours when this response was severe. In birds which survived the initial rise in blood uric acid, the normal level was restored in two to three days and at autopsy little or no uratic deposition was found (55). Marked muscular atrophy occurred. The rôle of dosage and the time factor have been discussed in connection with the differences in various birds (157). It is noteworthy that Dalmatian dogs, which are unable to oxidize uric acid, respond like other dogs by the development of diabetes and no abnormalities of uric acid metabolism have been reported in this breed (77). An increase in blood uric acid 48 hours after alloxan and the prevention of this phenomenon by colchicine has been reported (42).

Pathology of the Pancreas. Ordinarily there are no gross changes in the pancreas although congestion and hemorrhage is occasionally seen. The histological alterations have been fully described by the first observers (54–59) and other detailed accounts (11, 48) and reviews (51, 77, 101, 113) have confirmed their results. The review by Duff (51) is particularly recommended. The lesions are similar in the rabbit (6, 11, 26, 54, 56, 57, 86, 164), rat (7, 53, 58, 102, 127) and dog (26, 33, 48, 74, 166) so that a single description will suffice. In other species the changes have not been as fully reported and the reader is referred to the individual accounts (table 1). The resistance of some species to anatomical damage and the difficulty in defining the islet injury in others have already been noted.

Slight diminution of the granules in the nuclei and cytoplasm of the beta cells is detectable as early as 5 minutes after the injection of a diabetogenic dose of alloxan (11, 102). At 10 to 15 minutes this is definite and seems to affect first the beta cells at the centers of the larger islands. By one hour shrinkage of the injured cells is apparent and by two hours definite pyknosis of the nuclei is evident (11, 26, 53, 56). Nuclear pyknosis progresses and by three hours there may be some separation of the cells with homogeneous, eosinophilic cytoplasm. Thereafter there may be either

shrinking or swelling of the cells which coalesce as the necrosis advances. Pyknotic nuclei may remain for as long as 24 hours or more but evidence of karyolysis and complete disintegration and disappearance of cells may be observed from 5 hours onward (11). At 24 hours the centers of the islands are composed of pale staining debris with only shadows of nuclei or cell outlines recognizable. The time of final disappearance of the granules of the beta cells has ranged from 15 minutes (102) to 3 days (80). While these changes are proceeding in the beta cells, the alpha cells are often undamaged although they may show slight degenerative change (56). Descriptions of necrosis of occasional alpha cells (53, 56, 57, 102) and of the destruction of both alpha and beta cells in almost all of the islands (5, 7, 11) indicate the variable extent of the damage. Sometimes after prolonged diabetes it has been impossible to find any islet tissue at all (7, 58, 102), but usually a few islands composed largely or entirely of alpha cells remain. The final picture after diabetes has existed for a few weeks or more is that of striking atrophy of the islands. Thus, the late stages of alloxan diabetes are indistinguishable from the final appearance of the pancreas in pituitary diabetes in dogs.

The rapid necrosis of the islands after alloxan, like the slower destruction in partial pancreatectomy (1) or pituitary diabetes (135), is remarkable for the complete absence of cellular inflammatory reaction.

There is a wide variation in the injury of the islands produced by any one dose of alloxan. In addition, the anatomical response to varied doses has been studied. After small single doses the changes are of the same type but less extensive and severe (56). After massive doses necrosis is accelerated, the condition of the islands at 10 hours being comparable to islands at 24 hours after the usual diabetogenic dose (37). The response to repeated small doses has also been studied (7, 11, 51, 101). As acute changes are thus produced at different times the picture is complex, all stages of necrosis and atrophy appearing together with normal cells which have escaped injury (11). With daily doses of 25 mgm. per kgm. or less for two months there were abnormalities of the granules of the beta cells. The animals did not develop glycosuria but this agrees with the alterations in the glucose tolerance test under similar conditions (161). From the effects produced by small doses of alloxan, Hughes and Hughes (101) postulated a cycle of maturation in the islands. According to them alloxan in low dosage destroys the oldest beta cells which are normally in the centers of the largest islands. In normal rats the smallest beta cells were in the largest islands; after repeated doses of alloxan, the largest cells were in the largest islands. In the alloxan-resistant strain of hooded rats (51) repeated subdiabetogenic doses produced transitory diabetes with minimal lesions in the larger islands. Thereafter, the animals were resistant to the continued administration of alloxan.

Alloxan has not produced hyalinization or fibrosis of the islands, the lesions most commonly seen in human diabetes. Hydropic degeneration of the beta cells has been noted occasionally (6, 11, 48, 52, 54, 118). This is seen rarely in man and commonly in other types of experimental diabetes. Hydropic change has not occurred in rats, but in dogs (48) and rabbits (11, 52) it has been found after diabetes had been present for several weeks. This has been attributed to the effect of hyperglycemia on beta cells that had escaped destruction by alloxan (118). Mitotic figures have been only rarely observed in the surviving cells (7, 56, 58, 80) but it has been suggested that the

alpha cells may increase in number (11, 53, 80). The disintegration of the Golgi apparatus in the beta cells has been described (42). The timing of the histological changes in the first 24 hours may be related to the alterations in the blood sugar (11, 56). Pathologists agree that the injury from alloxan takes place within a few minutes of its injection and that the subsequent events are the consequence of this initial injury. Although there was "no alteration in the character of the lesion which could be correlated with the fall in blood sugar" (11), the changes which began immediately after the injection of alloxan had progressed to the complete disintegration of many cells by 6 hours, i.e., at the onset of the hypoglycemia phase.

The acinar tissue is commonly described as normal. Duff and Starr (53) observed numerous mitoses in the acinar cells at about 17 hours, but not thereafter. In the small intralobular ducts the hydropic change noted by Goldner and Gomori (74) has been confirmed (47, 52, 58, 93). Small foci of fat necrosis with the usual inflammatory reaction were found in 23 per cent of dogs by one group (47, 48, 93) who suggest that this was the result of more advanced injury of the small ducts with leakage of the external secretion. Grossman and Ivy (85) found a diminished threshold response to secretin 18 to 30 days after alloxan. The amylase activity of the secretion was normal. They suggested that this was due to the injury of the ducts which they confirmed and that therefore duct cells as well as acinar cells might participate in the formation of pancreatic juice.

Kidneys. The remarkable toxicity of alloxan for the beta cells of the islands of Langerhans is accompanied by damage of the renal tubules which may be due to the sensitivity of this tissue to the drug or to the increased concentration of the drug during excretion. Occasionally gross pallor of the cortex has been mentioned. The glomeruli are normal. The convoluted tubules are often the site of vacuolation or hydropic change, necrosis and desquamation of varying degree (7, 11, 26, 48, 53, 57, 58, 74, 93, 127, 154). Albuminuria and occasional casts may be present. The lesions have been compared to those of mercurial poisoning (93). Renal lesions are most conspicuous in the first four days after the administration of alloxan. Thereafter they tend to disappear (11, 80, 93, 127, 154) so that in late diabetes the kidney is usually normal. This corresponds to the changes in the nonprotein nitrogen of the blood which rises after the injection of alloxan and returns to normal in a few days if the animals survive (16, 127). Infiltration of leucocytes about the affected tubules is seldom seen (127). The relation of dosage to renal injury has been studied in the dog (74, 93) and rat (16, 127). As an example, Lazarow and Palay (127) found that after 40 mgm. per kgm. intravenously, 16 of 18 rats had no kidney injury whereas half of the animals given 200 mgm. per kgm. intraperitoneally showed moderate to severe changes. In diabetes of some duration glycogen deposition in the loops of Henle has been found (41, 80, 93). Curtis *et al.* (41) found that this occurred only if the terminal blood sugar was 350 mgm. per 100 ml. or higher. Glycogen deposition in the kidneys is a manifestation of diabetes and not of alloxan itself. Intercapillary glomerulosclerosis has not been reported. In summary, an acute necrosis of the renal tubules may follow appropriate doses of alloxan. If death from uremia does not occur these lesions are reversible, a fact in which the kidneys differ from the islands of Langerhans.

Liver. Although many writers report little or no abnormality of the liver in

alloxan diabetes (7, 11, 53, 80, 86), definite lesions have been observed by others. In an attempt to differentiate lesions caused by alloxan from those due to the ensuing diabetes, particular attention will be paid to the time after alloxan when the lesions were found. Thus, Goldner and Gomori (74) saw no hepatic lesions in dogs dying acutely from large doses of alloxan. Fatty infiltration, slight necrosis and increased phosphatase (by stain) were present in dogs diabetic for two weeks. In contrast, in dogs given large doses, Houssay *et al.* (93) described marked jaundice, positive cephalin-cholesterol flocculation tests and other signs of severe hepatic damage. They distinguish this from jaundice occurring late in 2 animals which they regarded as secondary to severe diabetic changes. They frequently found fatty infiltration and central lobular necrosis at three days or longer after alloxan. As fatty livers can develop in 48 hours in depancreatized dogs, the primary rôle of alloxan is not clear in these animals but the emphasis on necrosis may indicate an effect of the drug. In cats (154) the occurrence of small and large areas of hepatic necrosis and fatty degeneration, beginning at the third day, was not related to the severity or duration of diabetes. Focal necrosis has been seen with varying frequency in rats (80, 127, 146). The typical lesion is a small focus of necrosis usually located in the midzonal or peripheral region of the lobule. It is 45 to 200 micra in diameter, uniformly distributed and inflammatory reaction is slight or absent (127). After intraperitoneal alloxan, subcapsular lesions instead of diffuse foci were noted. The incidence and severity of hepatic involvement could not be related to the dosage of alloxan or to the occurrence of renal lesions. Some of the highest intravenous doses did not produce hepatic necrosis whereas some of the lower doses were accompanied by severe damage. Occasionally slight liver damage was encountered after nondiabetogenic doses (127). When cysteine was given intravenously with alloxan to protect the islets, the incidence of hepatic necrosis increased from 22 to 85 per cent. As cysteine alone was harmless this was attributed to the conversion of cysteine to cystine which is known to cause hepatic necrosis. Herbut' *et al.* (89) describe portal cirrhosis with iron deposition suggestive of hemochromatosis after alloxan in rabbits.

Other Organs. The adrenal glands increase in weight in alloxan (17) as in pancreatic (63) diabetes. The adrenal glands are usually described as normal or as having minor changes not regarded as significant (7, 11, 53, 57, 58, 74, 93). Congestion and hemorrhage have been seen (59). Hard and Carr (86) found necrosis of the medulla in animals given acutely toxic doses of alloxan. Lesions were slight or absent in diabetic animals examined one to three weeks after alloxan. Focal necrosis of the adrenal cortex, especially of the fascicular zone, has occurred with varying frequency (54, 117, 154, 164). In one report (117) cellular inflammatory reaction was noted. One gains the impression that injury of the adrenal gland, particularly of the cortex, may occur but that it is an unimportant factor in the routine use of animals with alloxan diabetes. The thyroid is atrophic (17) as in pancreatic diabetes (63), and the Golgi apparatus is reduced in size (2), changes which are attributed to the diabetes rather than to a direct effect of alloxan. The thyroid, parathyroid and pituitary glands are usually described as normal (11, 53, 57, 58). With large doses degenerative changes in the basophils of the pituitary have been seen (154, 164) and the intestinal absorption of glucose is greater than normal (147). Arteriosclerosis

has been sought but not found even in rabbits which are known to be sensitive to arterial injury (11, 51). The hypercholesterolemia of rabbits after alloxan is transitory unless the diabetes is severe (117). Three rabbits showed only a slight elevation at 50 to 60 days after alloxan so that the sustained diabetic hypercholesterolemia differs in degree from the extreme value produced by the forced feeding of cholesterol.

Alloxan in Man. Alloxan was first given to man by Brunschwig *et al.* (27) for the purpose of relieving hypoglycemia due to island cell carcinoma. They also gave it to 4 patients with severe malignant disease of other types. In the 4 persons with normal blood sugar there was a negligible hyperglycemic phase and no evidence of diabetes. The doses ranged from 50 to 500 mgm. per kgm., increasing amounts being given over periods up to six days. Because of this it is frequently asserted that man is resistant to alloxan. This may be so if one discounts the slower administration of the drug in 500 ml. of saline. In one patient they mention that the injection was completed in an hour and as Houssay *et al.* (93) point out slowing the injection of an otherwise effective dose to 10 minutes abolishes its diabetogenic action. One patient who received 600 mgm. per kgm. subsequently came to necropsy and minor lesions of the islands were found. The kidneys were entirely normal. One patient in this group had a chill, nausea and cyanosis for several hours after one of the large doses.

Five patients with hyperinsulinism have received alloxan. The first (27), who was known to have metastatic carcinoma of islet tissue, received several courses of alloxan which was given daily for one to four weeks. The dose was gradually increased to a maximum of 39 gm. in this 112-kgm. patient (348 mgm. per kgm.). After each course the patient had relief of hypoglycemic symptoms for periods as long as three weeks. At necropsy, the cells of the metastatic islet tumor showed no injury, the islets of the pancreatic tissue were described as normal and there was no tubular necrosis of the kidney. Another patient is that of Talbot, reported by Bailey (9, 113). In this 9-month-old girl with hypoglycemia, no tumor had been found at laparotomy and frequent convulsions continued to occur. Beginning at 20 mgm. per kgm. the dose was increased to 100 mgm. per kgm. over a period of seven days. Symptoms were relieved for three weeks and then recurred. A second series of 8 injections of 100 mgm. per kgm. was followed by freedom from symptoms for the eight months of observation reported. Although spontaneous remissions of hypoglycemia may occur this is a striking result. A third patient with hypoglycemia is briefly reported by Conn, Hinerman and Buxton (38). She was given 50 mgm. per kgm. on each of the first two days and 100 mgm. per kgm. daily for the next seven days, with temporary relief. However, after symptoms recurred an islet tumor was removed and normal pancreas was also secured for study. Histologically there was no effect of alloxan on the neoplastic islet cells, but marked destruction of the islet cell of the pancreas itself was observed. The patient was diabetic after the tumor was removed (37a). Finally, Wilder (171a) has mentioned a patient with hypoglycemia complicated by infection who died after alloxan with extensive fatty degeneration of the liver, and Flinn *et al.* (62) gave it to a patient with islet cell carcinoma.

Most of the injections of alloxan in man have caused no untoward effects. However, in addition to the reaction noted above, one of the hypoglycemic patients

(27) had five severe reactions on scattered occasions. The symptoms consisted of chill, nausea and vomiting sometimes followed by fever. On several occasions there was jaundice and severe anemia, the erythrocyte count falling as low as 1.7 million per cmm. Whether icterus was due to the hemolysis caused by alloxan or to direct injury of the liver is uncertain. Transfusions were used to aid the recovery of this anemia. A few casts, erythrocytes and a trace of albumin in the urine were transitory findings after a few injections.

Thus, two instances (27, 38) of the resistance of tumor cells and the susceptibility of normal human islets to alloxan are on record and another case of uncertain pathology may have been benefited. The reviewer agrees with Conn (37a) that "insufficient observations have been made to warrant the repeatedly expressed opinion that human islet tissue is exceedingly resistant to the degenerative effects of alloxan".

MECHANISM OF ACTION OF ALLOXAN

The conclusion that alloxan diabetes is pancreatic diabetes of varying degree seems so apparent from the correlation of both pathological and physiological data that this concept of *alloxan diabetes* will be accepted without further discussion. On the other hand, the fundamental action of alloxan on the islet cells is unknown and a summary of the investigations on this subject will be made. In the living animal alloxan necrosis of the beta cells results from the immediate action of an evanescent substance. The immediate action of alloxan means that it acts during the first five minutes or so after its rapid intravenous injection. This is supported by the findings that the earliest lesions appear in five minutes (11, 102), and that after this time alloxan is no longer demonstrable in the blood (3, 22, 115, 128). Goldner and Gomori (81) demonstrated this immediate action by clamping the circulation to the tail of the pancreas for five minutes during and after the administration of alloxan. In the clamped portion of the pancreas the islands were normal, whereas in the remainder of the organ the usual island necrosis occurred. Finally the chemical agents which protect the islands from alloxan (see below) must be given within a few minutes of the alloxan to be effective. After reviewing the chemical properties of alloxan, Archibald (3) came to the conclusion that there are at least three types of reaction which neutralize alloxan in blood: *a*) conversion to alloxanic acid by alkali, *b*) conversion to alloxantin and dialuric acid by the thiol groups in plasma and *c*) combination with urea.

Present evidence indicates that the action of alloxan on the islands is exerted directly. It is generally agreed that the pathological changes are the consequences of an immediate toxic injury of the beta cells. In addition, the mediation of certain other agents has been excluded. The initial hyperglycemic phase has been prevented by means of insulin (75, 76), phlorizin (75, 76), adrenalectomy (103, 119), hypophysectomy (10, 103, 119) and hepatectomy or evisceration (98, 99). In these experiments the occurrence of island injury has been reported often enough to make it clear that this initial period of hyperglycemia is not essential to the toxic action of alloxan on the islets. This deserves mention even though hyperglycemia of a few hours' duration would have no demonstrable effect on the islands, as days or weeks rather than hours are required to produce marked histological lesions in dogs or cats after

partial pancreatectomy (1), pituitary diabetes (135) or glucose administration (49). Moreover, hyperglycemia has not been accompanied by similar island lesions in the rat (46, 68), yet the rat is susceptible to alloxan. Alloxan necrosis and some degree of diabetes have been produced in the absence of the pituitary (7, 10, 51, 103) or adrenal glands (51) so that the organs which are, in varying degree, involved in other types of experimental diabetes play no part in the effect of alloxan. The sustained diabetes is not produced by inhibition of insulin because insulin is not inactivated by alloxan *in vitro* (75, 118) and because animals with alloxan diabetes respond normally to insulin (54, 74, 76, 93, 118). The action of alloxan on other tissues is apparently an acute injury of the parenchymal cells followed by necrosis or recovery depending on the conditions.

There are responses to alloxan which occur after its disappearance from the blood but which are relevant to its action on the islands and on the organism as a whole. Among these are the initial hyperglycemic and subsequent hypoglycemic phases of the blood sugar during the first 18 to 20 hours.

Hyperglycemic Phase. As noted above this may be prevented by various means without interfering with the necrosis of the islands. On the other hand, the mechanism by which this hyperglycemia is produced is still debatable. Dunn, Sheehan and McLetchie (59) suggested that this might be due to excessive mobilization of glucose through the adrenosympathetic system. This hypothesis has received support from subsequent studies. In adrenalectomized rabbits (75) and rats (51, 75, 119) and in rabbits with the adrenal medullae destroyed (75), the hyperglycemic phase was abolished. Corkill *et al.* (39) found that blocking the sympathetic nervous system by ergotoxine abolished the hyperglycemia, and Hughes, Ware and Young (102) duplicated both phases of the blood sugar response to alloxan by the combined administration of epinephrine and insulin. All of these results support the concept that the hyperglycemic phase is a non-specific response mediated by the sympathetic system. However there are other observations which show that the question is not settled. Houssay *et al.* (98, 99) found hyperglycemia in dogs (98, 99) and toads (96) after adrenalectomy. The reviewer would conclude from their data (98) that normal dogs under chloralose had a small hyperglycemic response to alloxan and that this was absent after adrenalectomy or denervation. The hyperglycemic phase is less in dogs than in rabbits (26). Houssay's interpretation is supported by others who have found that the hyperglycemic phase was not prevented by ablation of the adrenal medullae or sympathectomy (103, 119, 148, 159, 162). When alloxan was injected via the portal vein (98, 99) the hyperglycemia was higher than in the control series so that the possibility of a direct influence of the liver on this phase of the blood sugar curve must be kept in mind. The results of Goldner and Gomori (75) have been criticized by Iverson (103) who concluded that the cortex, rather than the medulla, is responsible for the effects of adrenalectomy. A final understanding of the hyperglycemic phase must await further study.

Hypoglycemic Phase. This has been attributed to *a*) an insulin-like action of alloxan (104), *b*) a transitory stimulation of the beta cells (59), *c*) the release of preformed pancreatic insulin (102) and *d*) an effect of alloxan directly on the liver (99). There is now ample evidence that alloxan has no insulin-like activity as Jacobs (104)

assumed, nor has it any effect on the utilization of glucose (39). The histologic changes in the pancreas are not consistent with the concept of a temporary overactivity of the beta cells. At the time of the hypoglycemia, 6–12 hours after alloxan, the beta cells are in a fairly advanced stage or necrosis. Incidentally it may be that there is an element of stimulation of the islands at a much earlier time if one recalls the initial fall in blood sugar at 15 minutes described by Shipley and Beyer (159), and the occasional mitoses observed after small doses (56) and discussed by Berthoud (19). Most reviewers agree that stimulation of the islands is not the explanation of the hypoglycemic phase.

The most generally accepted hypothesis is that preformed insulin is liberated from the damaged islands. This is supported by the general course of the histological lesions, the knowledge that alloxan does not neutralize insulin *in vitro* (75, 118), and the reproduction of the hypoglycemia with a dose of insulin corresponding to the insulin content of the animal's pancreas (102). Moreover there has been no fall in the blood sugar when alloxan is administered to alloxan-diabetic animals (77, 118) or to depancreatized animals (13, 79) and the insulin content of the pancreas does not begin to diminish until the necrosis of the beta cells is well advanced (7–8 hours), this diminution of insulin content coinciding approximately with the development of hypoglycemia (151).

There has been some disagreement with portions of the evidence cited above. In contrast to Banerjee (13) who used rabbits, Foglia, Orias and Sara (65) using rats reported the usual hypoglycemia after partial pancreatectomy. Although Goldner and Gomori (77, 79) found no hypoglycemia in depancreatized dogs, Houssay, Orias and Sara (99) observed a fall in blood sugar. This was found in dogs depancreatized one-half hour before the alloxan was given, but if 24 hours or more elapsed between pancreatectomy and the administration of alloxan they found, like others, no effect of alloxan on the blood sugar. They interpret this to mean that the liver becomes insensitive to alloxan. Another and most important explanation of these differences is made by Best and his associates (20). They have repeated the experiments with alloxan in depancreatized dogs using two preparations of alloxan. With the alloxan used by Goldner and Gomori (79) they obtained results in agreement with the Chicago investigators, and their results agreed with Houssay's (99) when Houssay's alloxan was employed. Pending further clarification, it seems that the hypoglycemic phase of the blood sugar after the administration of alloxan is due to the liberation of insulin from the necrotic island cells to which an effect of alloxan on the liver may be added when certain preparations of alloxan are used.

Partially depancreatized rats (145) are more resistant than normal controls to the diabetogenic effect of single doses of alloxan. Partially depancreatized dogs (93) also require the full dose. However, the depancreatized rats are more susceptible than normal rats to repeated low doses of alloxan. This indicates that it is the concentration of alloxan in the blood, rather than the mass of islet tissue, which determines the effect of a single dose. On the other hand, the gradual injury of islet tissue by repeated doses is more readily revealed when the insular reserve is reduced. When diabetes is established 24 hours or more after an effective dose of alloxan, the insulin content of the pancreas falls to a very low level (75, 151). At this stage the secretion

of insulin, measured by grafting the diabetic pancreas in a depancreatized dog, was usually greatly reduced (99).

Houssay *et al.* (96) found that the diabetogenic action of the pituitaries of alloxan-treated toads seemed slightly diminished. In rats (94) there was no such diminution and all pituitary hormones appeared normal except for a reduction in gonadotrophic activity. They relate this result to the minor lesions described by Thomas and Emerson (164) in the basophilic cells which are thought to elaborate the gonadotrophic hormone. In established alloxan diabetes, where the mechanism of alloxan itself is no longer an issue, hypophysectomy in both rats (10) and rabbits (103) leads to a somewhat milder and unstable type of diabetes reminiscent of the results seen in the 'Houssay' dog. Adrenalectomized rats are more sensitive to the hypoglycemic effect of alloxan as would be expected (140). Adrenalectomy caused an amelioration of alloxan diabetes in rats (106, 107) and a fall in liver glycogen to 15 per cent of that in control diabetic animals. In both hypophysectomy and adrenalectomy the decline in food intake is an important variable which must be controlled (103, 106). Pretreatment with estrogens does not alter the blood sugar curve after the injection of alloxan (103). Established diabetes is not notably influenced by estrogenic therapy (103, 105) when the alterations in food intake are considered. When food intake is maintained by tube feeding estrogens made the diabetes more severe (102a). Estrogens caused some increase in glycogen levels of diabetic rat (97).

Protection from Alloxan Diabetes. This has been accomplished by a variety of agents *provided the protective substance is given almost simultaneously with the alloxan.* Benerjee (14) found that the following substances given mmediately before alloxan prevented the development of diabetes in rabbits: 1,2-dimethyl-4-amino-5 (d-l-ribityl-amino)-benzene; nicotinic acid; pyridine-dicarboxylic acid and 2-phenyl-quinoline-4-carboxylic acid. He noted that these compounds did not prevent the initial hyperglycemia although they abolished the hypoglycemia and ultimate diabetes. He found (14) that the following substances did not prevent diabetes: calcium pantothenate, glycine, benzoic acid, p-amino-benzoic acid, ascorbic acid and pyridoxine hydrochloride. The 1,2-dimethyl-4-amino-5 (d-l- ribityl-amino)-benzene was the reagent used to react with alloxan to form riboflavin (15) and this was thought to be the mechanism of its protection *in vivo.* Nicotinic acid was studied accidentally and the other compounds were tried in an effort to explain its action. He concluded that neither the carboxyl group nor the pyridine ring was responsible and that the entire nicotinic acid molecule was involved. The first reported attempt to prevent alloxan diabetes was that of Leech and Bailey (128) who used glutathione. This followed their observation (128) that about five minutes after the intravenous injection of alloxan the blood glutathione falls, often to zero, and a fall in blood thioneine was also found. The fall in glutathione has been confirmed (25). However, glutathione in doses of 200 mgm. per kgm. failed to protect rabbits. Lazarow (126) using 2500 mgm. per kgm. of glutathione and 912 mgm. per kgm. of cysteine found that both prevented diabetes in rats. Alanine, glycine, phosphate buffer, ascorbic acid and succinic acid did not protect. If the sulfhydryl compounds were given three minutes after the alloxan injection instead of two minutes before it, there was no pro-

tection. In additional studies on the prevention of diabetes by cysteine, a marked increase in the incidence of necrosis of the liver occurred after cysteine even though the islands were protected (146). Weinglass, Frame and Williams (170) protected rabbits from the hypoglycemic and diabetogenic effects of alloxan by immediate pretreatment with 3,4-diamotoluene, orthophenylenediamine and sodium bisulfite. They found the following ineffective: sodium bicarbonate, sodium phosphates (pH 7.0), sodium pyrophosphate, sodium cyanide, phenylhydrazine, hydroxylamine hydrochloride, cysteine hydrochloride, paraphenylenediamene, sodium pyrogallol and lysine hydrochloride. Colchicine inhibits the second hyperglycemic phase and prevents the increase in blood uric acid which occurs about 48 hours in rats given alloxan (42). The prophylactic influence of ribonucleotides has been reported (61). Ascorbic acid, already referred to as having no preventive action, was regarded as increasing the diabetogenic action of alloxan (130). This was not observed after d-isoascorbic acid. British Anti-Lewisite (BAL) gave complete protection in rats (37, 126a).

The prevention of alloxan diabetes by certain physiological procedures has been the subject of several studies. The rôle of diet and the protection of a portion of the pancreas by clamping its circulation for a short time have been noted. Walpole and Innes (168) ligated the pancreatic ducts and 30 to 60 days later, when there was pancreatic sclerosis with persistence of normal islet tissue, injected alloxan. Under their conditions alloxan had little or no pathological effect on the islets and did not produce diabetes. In constrast, Goldner and Gomori (79) saw no protection against the degenerative action of alloxan after fibrosis of the pancreas had been produced in this manner. The discrepancy between these investigators is still unexplained except for the fact that Goldner and Gomori used dogs instead of rabbits. Jimenez-Diaz *et al.* (111) described complete protection of dogs from the diabetogenic and nephrotoxic effects of alloxan if the circulation to the kidneys was clamped during the time necessary for the inactivation of alloxan in the blood. They interpret this as indicating some rôle of the kidneys in the diabetogenic effect of alloxan. Both the clamped and control animals had the initial hyperglycemic and the hypoglycemic response but the clamped ones did not develop diabetes. Confirmation of this work has not yet appeared. Thymectomy increased the susceptibility of rats to alloxan although splenectomy or castration had no such effect (61). Houssay *et al.* (92, 95, 100) and Martinez (137, 138, 139) have studied the influence of the thyroid on both alloxan and pancreatic diabetes in the rat. Thyroidectomized animals were more resistant and thyroid-treated animals were more sensitive to intravenous alloxan, LD_{50} being 74 and 25 mgm. per kgm., respectively. Thiouracil caused even more resistance to alloxan than thyroidectomy. In subtotally depancreatized rats thyroid treatment caused an earlier appearance of diabetes but led to the subsequent disappearance of diabetes, a result attributed to the hyperplasia of the islets due to the thyroid treatment. They point out the differences between the dog and the rat in respect to this response of the islands (95). In summary, protection from the diabetogenic action of alloxan has been demonstrated by: *a*) substances which combine directly with alloxan, i.e., they do so *in vitro* and presumably do so on injection in animals; *b*) physiological conditions under which the inactivation of alloxan is facili-

tated by means not yet understood. These experiments tell one little about the fundamental action of alloxan although the results with most of the chemical antagonists emphasize the rapid and transitory nature of its action.

Action of Alloxan Homologues and Related Compounds. Another approach to the question of the mechanism by which alloxan destroys the beta cells is the determination of the chemical specificity of its effect. This has received considerable attention and tables 4 and 5 indicate the results. Table 6 supplements these listings with a few structural formulae. In table 4 it will be seen that some compounds have been found to be diabetogenic with a high degree of uniformity. Initial experiments (e.g., 75) which were negative may be attributed to technical difficulties and it is easy to reach the conclusion that under appropriate conditions all of the compounds listed, except barbituric acid and violuric acid, are diabetogenic. None is as readily effective as alloxan itself. In the case of barbituric and violuric acids it is possible that a positive result is more significant than the failures to produce island lesions. Further

TABLE 4. OTHER COMPOUNDS REPORTED AS DIABETOGENIC

SUBSTANCE	DIABETES REPORTED	DIABETES NOT REPORTED
Monomethyl-alloxan	(24, 25, 71, 90)	
Monoethyl-alloxan	(25)	
Monopropyl-alloxan	(25)	
Alloxantin	(7, 24, 25, 83, 88, 91)	(75)
Dimethyl-alloxantin	(24, 25)	
Diethyl-alloxantin	(24, 25)	
Dialuric acid	(8, 24, 25, 83, 121)	(75)
Monomethyl-dialuric acid	(24, 25)	
Barbituric acid	(71)	(25, 75, 83, 121)
Violuric acid	(71)	(24, 25, 75, 83, 165)

study of these substances is needed. The long list of compounds without diabetogenic action (table 5) requires only the comment that this list is neither complete nor final. That it is not necessarily the last word is illustrated by the report of Stoll (163) that ninhydrin was diabetogenic. The hypoglycemic effect of sulfanilamide-cyclopropylthiazole was not accompanied by diabetes (35).

The most recent discussion of the data in tables 4 and 5 is that of Brückmann and Wertheimer (25) which is the basis of the following remarks. An intact pyrimidine nucleus appears essential for diabetogenic activity. Some inactive substances like dimethylalloxan still produce kidney injury which may mean that the kidney damage is not due to the same mechanism as the island damage. These authors suggest the following possible types of action to account for the direct effect on the islet cells. 1) There may be selective accumulation of the drug in the beta cells. Because of the small amount of islet tissue this is possible in spite of their finding that after its injection there is less alloxan in the pancreas than in the liver and kidney.

2) Alloxan may compete with some structurally similar compound for an enzyme with resulting injury to the cells. This is entirely speculative at present. 3) A specific reaction of alloxan may occur in the islet cells with or without a specific accumulation. Lazarow (126) has suggested that the inactivation of SH groups of protein enzymes might account for such an effect. In view of the large reserve of glutathione

TABLE 5. COMPOUNDS WITHOUT DIABETOGENIC ACTION

COMPOUND	REFERENCES	COMPOUND	REFERENCES
Allantoin	(71)	Oxaluric acid	(104)
		Formyl-oxaluric acid	(104)
Alloxanic acid	(24)		
Dimethyl-alloxanic acid	(24)	Parabanic acid	(71, 165)
Benzal-barbituric acid	(165)	Pipe azine	(71)
Benzoyleneurea	(90)		
		Rhodizonic acid	(25)
Benzyl-alloxan	(25)		
Butyl-alloxan	(25)	Senecionene	(87)
Isobutyl-alloxan	(25)		
Phenyl-alloxan	(25)	Sulfadiazine	(87)
Dimethyl-alloxan	(25, 90)	Tartronic acid	(24)
Methyl-ethyl-alloxan	(25)		
Methyl-propyl-alloxan	(25)	Thiouracil	(64, 165)
Dimethyl-dialuric acid	(25)	Uracil	(71)
Isodialuric acid	(104)		
		Uramil	(71, 165)
Formyl urea	(104)	N-methyl-uramil	(25)
Guanidin	(57)	Uric acid	(57, 71, 165)
Isatin	(24, 25, 90)	Other oxidizing substances	(75)
		Ceric sulfate	(75)
Isobarbituric acid	(104)	Persulfate	(75)
N-dodecyl barbituric acid	(75)	Quinone	(75)
		Sodium molybdate	(75)
Mesoxalic acid	(104)		
Sodium mesoxalate	(90)	Related to styryl quinoline	(55)
Ethyl mesoxalate	(90)	1,2-naphthoquinone-4-sulfonic acid	(25)
Mesoxalamide	(90)	Naphthoquinone	(75)
		1,8, mesoxalylnaphthalene mesoxalyl	(25)
Murexide	(24)	Quinoline	(75)
		Cincophen	(75)
Ninhydrin	(24, 25, 90)		
Oxalic acid	(57)		

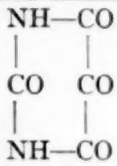
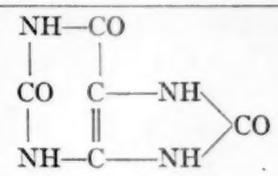
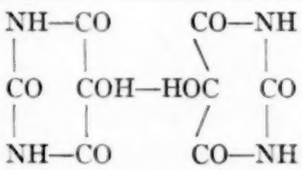
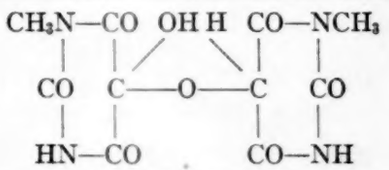
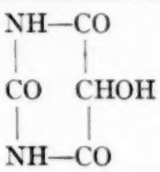
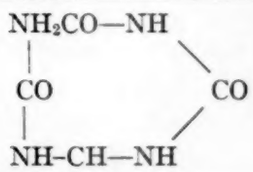
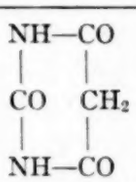
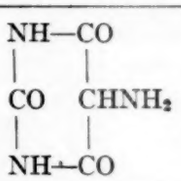
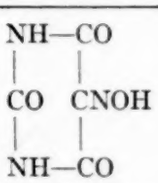
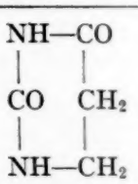
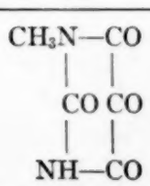
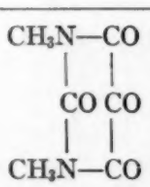
in the pancreas after alloxan (25) and the relatively unspecific nature of SH oxidation this concept is doubted. Brückmann and Wertheimer point out that lack of knowledge of the biochemical processes in the islands of Langerhans is the major barrier to any final understanding of the action of alloxan.

Possible Relation of Alloxan to Enzymatic Reactions. In reviewing the few

references in this field it is necessary to distinguish between the direct effects of alloxan and studies in established alloxan diabetes. The latter give no information about the primary action of alloxan and are included only for the sake of completeness.

Reference has already been made to the physical chemistry of the alloxan dialuric acid oxidation: reduction system (150, 171) and to the reversible reaction: uric acid-dialuric acid in the perfused liver (4). In appropriate concentrations alloxan in-

TABLE 6. CHEMICAL STRUCTURE OF ALLOXAN AND RELATED COMPOUNDS

Alloxan		Uric acid	
Alloxantin		Dimethyl-alloxantin	
Dialuric acid		Allantoin	
Barbituric acid		Uramil	
Violuric acid		Uracil	
Methyl alloxan		Dimethyl alloxan	

creased the oxygen uptake of liver suspensions and greatly accelerated their oxidation of ethyl alcohol (18). Alloxan had no direct effect on hepatic glycogenolysis (31) and it inhibited the glycolysis of glycogen to lactate in frog muscle extract and this inhibition was reversed by cysteine (70). This agrees with previous work on the inhibition of hexosephosphate formation from glycogen by alloxan (129). In experiments timed from 6 to 48 hours after alloxan, there was a diminution in the alkaline phosphatase of the kidney (141), which was also demonstrated histochemically.

Serum diastase was temporarily increased 6 hours after alloxan but returned to the normal level by the time permanent hyperglycemia was established (66).

Rats with established alloxan diabetes have been used to study the oxidation of various substrates by brain, liver and kidney *in vitro* (32). There were no differences between the normal and diabetic tissues in these studies and in contrast to earlier work (18) alloxan did not increase the oxygen uptake of liver. Increased glycogen phosphorylysis in muscle (125) and an increase in liver (50, 74) and serum (30, 125) phosphatase all point to the splitting of phosphate esters at certain stages in alloxan diabetes. As noted, studies made after the initial effects of alloxan have disappeared are not apparently related to its mode of action. For this reason major contributions to carbohydrate metabolism in which alloxan diabetic rats have been used are not discussed.

SUMMARY

Alloxan diabetes is the diabetes produced by the administration of alloxan in various ways to many species of animals. It is the result of the immediate action of alloxan on the islet cells, especially the beta cells, which results in their necrosis with subsequent atrophy of the islands. The permanent diabetes is therefore a form of pancreatic diabetes, i.e., it is partial pancreatectomy of varying degree which has been chemically induced. The acinar tissue is uninjured. Alloxan as such disappears from blood *in vitro* and *in vivo* in 10 minutes or less and has not been found in the blood in human diabetes. From its pathology and physiology alloxan diabetes is due to the destruction of islet tissue but the mechanism by which it injures the cells is unexplained. Alloxan diabetes differs strikingly from the other types of experimental diabetes in the course and nature of the lesions which are developed. It is a valuable aid in the study of diabetes and it may open the way to increased knowledge of the function of the cells of the islands of Langerhans. As yet there is no evidence that alloxan is related to human diabetes mellitus.

REFERENCES

1. ALLEN, F. M. *J. Metab. Res.* 1: 5, 1922.
2. APPLEGARTH, A. P. AND A. A. KONEFF. *Anat. Rec.* 96: 13, 1946.
3. ARCHIBALD, R. M. *J. Biol. Chem.* 158: 347, 1945.
4. ASCOLI, M. AND G. IZAR. *Ztschr. physiol. chem.* 62: 347, 1909.
5. BAILEY, C. C. AND O. T. BAILEY. *J. Am. Med. Assoc.* 122: 1165, 1943.
6. BAILEY, C. C., O. T. BAILEY AND R. S. LEECH. *New England J. Med.* 230: 533, 1944.
7. BAILEY, C. C., O. T. BAILEY AND R. S. LEECH. *Bull. New England Med. Center.* 7: 59, 1945.
8. BAILEY, C. C., O. T. BAILEY AND R. S. LEECH. *Proc. Soc. Exper. Biol. and Med.* 63: 502, 1946.
9. BAILEY, C. C. AND P. LECOMPTE. *Med. Clin. North America.* 31: 427, 1947.
10. BAILEY, C. C., P. LECOMPTE, O. T. BAILEY AND C. C. FRANSEEN. *Proc. Soc. Exper. Biol. and Med.* 66: 271, 1947.
11. BAILEY, O. T., C. C. BAILEY AND W. H. HAGAN. *Am. J. Med. Sci.* 208: 450, 1944.
12. BANERJEE, S. *Lancet* 2: 658, 1944.
13. BANERJEE, S. *J. Biol. Chem.* 158: 547, 1945.
14. BANERJEE, S. *Science* 106: 128, 1947.
15. BANERJEE, S., K. DITMER AND V. A. DU VIGNEAUD. *Science* 101: 647, 1945.
16. BENNETT, L. L. AND T. BEHRENS. *Proc. Soc. Exper. Biol. and Med.* 62: 5, 1946.
17. BENNETT, L. L. AND A. A. KONEFF. *Anat. Rec.* 96: 1, 1946.

18. BERNHEIM, F. *J. Biol. Chem.* 123: 741, 1938.
19. BERTHOUD, E. *Presse Med.* 54: 875, 1946.
20. BEST, C. H. Personal communication.
21. BIASOTTI, A. AND J. PORTO. *Rev. Soc. argent. de biol.* 21: 63, 1945.
22. BRÜCKMANN, G. *J. Biol. Chem.* 165: 103, 1946.
23. BRÜCKMANN, G. *Endocrinology* 41: 201, 1947.
24. BRÜCKMANN, G. AND E. WERTHEIMER. *Nature* 155: 267, 1945.
25. BRÜCKMANN, G. AND E. WERTHEIMER. *J. Biol. Chem.* 168: 241, 1947.
26. BRUNSCHWIG, A. AND J. G. ALLEN. *Cancer Res.* 4: 45, 1944.
27. BRUNSCHWIG, A., J. G. ALLEN, F. M. OWENS AND T. F. THORNTON. *J. Am. Med. Assoc.* 124: 212, 1944.
28. BURN, J. H., T. H. C. LEWIS AND F. D. KELSEY. *Brit. Med. J.* 2: 752, 1944.
29. CANDELA, J. L. R. *Rev. Clin. Esp.* 19: 326, 1945.
30. CANTOR, M. M., J. TUBA AND P. A. CAPSEY. *Science* 105: 476, 1947.
31. CANZANELLI, A., R. GUILD AND D. RAPPORT. *Science* 104: 223, 1946.
32. CANZANELLI, A., R. GUILD AND D. RAPPORT. *Endocrinology* 38: 245, 1946.
33. CARRASCO-FORMIGUERA, R. *J. Lab. Clin. Med.* 29: 510, 1944.
34. CERECEDO, L. R. *J. Biol. Chem.* 93: 283, 1931.
35. CHEN, K. K., R. C. ANDERSON AND N. MAZE. *Proc. Soc. Exper. Biol. and Med.* 63: 483, 1946.
36. CHESLER, A. AND R. TISLOWITZ. *Science* 101: 468, 1945.
37. CHESLER, A. AND R. TISLOWITZ. *Science* 106: 345, 1947.
37a. CONN, J. W. *Proc. Am. Diabetes Assoc.* 6: 391, 1946.
38. CONN, J. W., D. L. HINERMAN AND R. W. BUXTON. *J. Lab. and Clin. Med.* 32: 347, 1947.
39. CORKILL, A. B., P. FANTL AND J. F. NELSON. *Med. J. Australia* 31: 285, 1944.
40. CRAMER, F. K. AND V. G. FOGLIA. *Rev. Soc. argent. de biol.* 19: 483, 1943.
41. CURTIS, G. W., S. L. ROBBINS AND I. GLICKMAN. *J. Exper. Med.* 85: 373, 1947.
42. CUTTING, W. C. AND G. L. LAQUER. *Stanford Med. Bull.* 4: 48, 1946.
43. DAVIS, M. E., N. W. FUGO AND K. G. LAWRENCE. *Federation Proc.* 6: 32, 1947.
44. DAVIS, M. E., N. W. FUGO AND K. G. LAWRENCE. *Proc. Soc. Exper. Biol. and Med.* 66: 638, 1947.
45. DE OYA, J. C. AND F. GRANDE-COVIAN. *Rev. Clin. Esp.* 16: 412, 1945.
46. DE ROBERTIS, E. *Rev. Soc. argent. de biol.* 21: 273, 1945.
47. DI PIETRO, A. AND A. F. CARDEZA. *Rev. Soc. argent. de biol.* 22: 288, 1946.
48. DI PIETRO, A. AND A. F. CARDEZA. *Arch. Soc. argent. de Anat. norm. y path.* 8: 1, 1946.
49. DOHAN, F. C. AND F. D. W. LUKENS. *Science* 105: 183, 1947.
50. DRABKIN, D. L. AND J. B. MARSH. *J. Biol. Chem.* 168: 777, 1947.
51. DUFF, G. L. *Am. J. Med. Sci.* 210: 381, 1945.
52. DUFF, G. L., G. MCMILLAN AND D. C. WILSON. *Proc. Soc. Exper. Biol. and Med.* 64: 251, 1947.
53. DUFF, G. L. AND H. STARR. *Proc. Soc. Exper. Biol. and Med.* 57: 280, 1944.
54. DUFFY, E. *J. Path. and Bact.* 57: 199, 1945.
55. DUNN, J. S. *Lancet* 1: 664, 1943.
56. DUNN, J. S., E. DUFFY, M. K. GILMOUR, J. KIRKPATRICK AND N. G. B. MCLETCHIE. *J. Physiol.* 103: 233, 1944.
57. DUNN, J. S., J. KIRKPATRICK, N. G. B. MCLETCHIE AND S. V. TELFER. *J. Path. and Bact.* 55: 245, 1943.
58. DUNN, J. S. AND N. G. B. MCLETCHIE. *Lancet* 2: 384, 1943.
59. DUNN, J. S., H. L. SHEEHAN AND N. G. B. MCLETCHIE. *Lancet* 1: 484, 1943.
60. DYE, J. A. AND B. A. WOODWARD. *Federation Proc.* 6: 99, 1947.
61. EMERSON, G. A., P. L. EWING AND T. B. THOMAS. *Federation Proc.* 6: 328, 1947.
62. FLINN, L. B., E. MINNICK AND D. M. GAY. *Ann. Int. Med.* 26: 936, 1947.
63. FOGLIA, V. G. *Rev. Soc. argent. de biol.* 21: 45, 1945.
64. FOGLIA, V. G. AND F. K. CRAMER. *Proc. Soc. Exper. Biol. and Med.* 55: 218, 1944.
65. FOGLIA, V. G., O. ORIAS AND J. G. SARA. *Rev. Soc. argent. de biol.* 20: 440, 1944.

66. FREE, A. H., E. M. ZORN AND V. C. MYERS. *Federation Proc.* 4: 91, 1945.
67. FRIEDGOOD, C. E. AND A. A. MILLER. *Proc. Soc. Exper. Biol. and Med.* 59: 62, 1945.
68. FRIEDMAN, N. B. AND A. MARBLE. *Endocrinology* 29: 577, 1941.
69. GARCIA RAMOS, J. *Rev. Soc. Mexicana de Hist. Nat.* 5: 25, 1944.
70. GEMMILL, C. L. *Am. J. Physiol.* 150: 613, 1947.
71. GITTER, S. AND A. F. CARDEZA. *Rev. Soc. argent. de biol.* 22: 353, 1946.
72. GITTER, S. AND H. PRIETO DIAZ. *Rev. Soc. argent. de biol.* 22: 1, 1946.
73. GOLDNER, M. G. *Bull. N. Y. Acad. Med.* 21: 44, 1945.
74. GOLDNER, M. G. AND G. GOMORI. *Endocrinology* 33: 297, 1943.
75. GOLDNER, M. G. AND G. GOMORI. *Endocrinology* 35: 241, 1944.
76. GOLDNER, M. G. AND G. GOMORI. *Proc. Soc. Exper. Biol. and Med.* 55: 73, 1944.
77. GOLDNER, M. G. AND G. GOMORI. *Proc. Am. Diabetes Assoc.* 4: 89, 1944.
78. GOLDNER, M. G. AND G. GOMORI. *Proc. Soc. Exper. Biol. and Med.* 58: 31, 1945.
79. GOLDNER, M. G. AND G. GOMORI. *Proc. Soc. Exper. Biol. and Med.* 65: 18, 1947.
80. GOMORI, G. AMD M. G. GOLDNER. *Proc. Soc. Exper. Biol. and Med.* 54: 287, 1943.
81. GOMORI, G. AND M. G. GOLDNER. *Proc. Soc. Exper. Biol. and Med.* 58: 232, 1945.
82. GRANDE-COVIAN, F. AND J. C. DE OYA. *Rev. Clin. Esp.* 15: 262, 1944.
83. GRANDE-COVIAN, F. AND J. C. DE OYA. *Rev. Clin. Esp.* 17: 320, 1945. See *J. Am. Med. Assoc.* 133: 496, 1947.
84. GRANDE-COVIAN, F. AND J. C. DE OYA. *Rev. Clin. Esp.* 19: 243, 1946.
85. GROSSMAN, M. L. AND A. C. IVY. *Proc. Soc. Exper. Biol. and Med.* 63: 62, 1946.
86. HARD, W. L. AND C. J. CARR. *Proc. Soc. Exper. Biol. and Med.* 55: 214, 1944.
87. HARRIS, P. N., R. C. ANDERSON AND K. K. CHEN. *J. Pharmacol. and Exper. Therap.* 87: 382, 1946.
88. HERBUT, P. A., J. S. WATSON AND E. PERKINS. *Arch. Path.* 42: 214, 1946.
89. HERBUT, P. A., J. S. WATSON AND E. PERKINS. *Am. J. Clin. Path.* 16: 506, 1946.
90. HIDY, H. P. *J. Biol. Chem.* 163: 307, 1946.
91. HOUSSAY, B. A. *New England J. Med.* 214: 961 and 971, 1936.
92. HOUSSAY, B. A., R. F. BRIGNONE, A. F. CARDEZA AND J. G. SARA. *Rev. Assoc. med. argent* 22: 241, 1946.
93. HOUSSAY, B. A., R. F. BRIGNONE AND P. MAZOCCO. *Rev. Soc. argent. biol.* 22: 195, 1946.
94. HOUSSAY, B. A. AND V. G. FOGLIA. *Rev. Soc. argent. biol.* 22: 329, 1946.
95. HOUSSAY, B. A., V. G. FOGLIA AND C. MARTINEZ. *Endocrinology* 39: 361, 1946.
96. HOUSSAY, B. A., A. B. HOUSSAY AND J. G. SARA. *Rev. Soc. argent. biol.* 21: 74, 1945.
97. HOUSSAY, B. A. AND P. MAZOCCO. *Rev. Soc. argent. biol.* 22: 367, 1946.
98. HOUSSAY, B. A., O. ORIAS AND J. G. SARA. *Rev. Soc. argent. biol.* 21: 30, 1945.
99. HOUSSAY, B. A., O. ORIAS AND I. SARA. *Science* 102: 197, 1945.
100. HOUSSAY, B. A. AND J. G. SARA. *Rev. Soc. argent. biol.* 21: 81, 1945.
101. HUGHES, H. AND G. E. HUGHES. *Brit. J. Exper. Path.* 25: 126, 1944.
102. HUGHES, H., L. L. WARE AND F. G. YOUNG. *Lancet* 1: 148, 1944.
102a. INGLE, D. J., J. E. NEZAMIS AND M. C. PRESTRUD. *Endocrinology* 41: 207, 1947.
103. IVERSON, M. *Reports of the Steno Memorial Hosp.* Copenhagen, 1947. Vol. II.
104. JACOBS, H. R. *Proc. Soc. Exper. Biol. and Med.* 37: 407, 1937.
104a. JANES, R. G. *Endocrinology* 40: 458, 1947.
105. JANES, R. G. AND H. DAWSON. *Endocrinology* 38: 10, 1946.
106. JANES, R. G., H. DAWSON AND L. MYERS. *Am. J. Physiol.* 145: 538, 1946.
107. JANES, R. G. AND C. E. FRIEDGOOD. *Endocrinology* 36: 62, 1945.
108. JANES, R. G. AND L. MYERS. *Proc. Soc. Exper. Biol. and Med.* 63: 410, 1946.
109. JARRETT, I. G. *Nature* 157: 441, 1946.
110. JARRETT, I. G. *Australian J. Exper. Biol. and Med. Sci.* 24: 95, 1946.
111. JIMENEZ-DIAZ, C., F. GRANDE COVIAN AND J. C. DE OYA. *Nature* 158: 589, 1946.
112. JOSLIN, E. P. *New England J. Med.* 230: 425, 1944.
113. JOSLIN, E. P., H. F. ROOT, A. MARBLE AND C. C. BAILEY. *Treatment of Diabetes Mellitus* (8th ed.). Philadelphia: Lea and Febiger, 1946.
114. KAPLAN, N. O., M. FRANKS AND C. E. FRIEDGOOD. *Science* 102: 447, 1945.

115. KARRER, P., F. KOLLER AND H. STURZINGER. *Helv. Chim. Acta* 28: 1529, 1945.
116. KASS, E. H. AND B. A. WASIBREN. *Proc. Soc. Exper. Biol. and Med.* 60: 303, 1945.
117. KENDALL, F. E., W. MEYER, L. LEWIS AND J. VICTOR. *Proc. Soc. Exper. Biol. and Med.* 60: 190, 1945.
118. KENNEDY, W. B. AND F. D. W. LUKENS. *Proc. Soc. Exper. Biol. and Med.* 57: 143, 1944.
119. KIRSCHBAUM, A., L. J. WELLS AND D. MOLANDER. *Proc. Soc. Exper. Biol. and Med.* 58: 294, 1945.
120. KOREF, O., L. VARGOS, JR., F. H. RODRIGUEZ AND A. T. TELCHI. *Endocrinology* 35: 391, 1944.
121. KOREF, O., L. VARGOS, JR., AND A. T. TELCHI. *Anal. Soc. Biol., Bogota* 2: 92, 1946.
122. LABES, R. AND H. FREISBURGER. *Arch. f. exper. Path. u. Pharm.* 156: 226, 1930.
123. LACKEY, R. W., C. A. BRINDLE, A. J. GILL AND L. C. HARRIS. *Proc. Soc. Exper. Biol. and Med.* 57: 191, 1944.
124. LANG, G. *Wien. med. Wchnschr.* 16: 1512, 1866.
125. LASZT, L. AND H. VOGEL. *Nature* 158: 588, 1946.
126. LAZAROW, A. *Proc. Soc. Exper. Biol. and Med.* 61: 441, 1946.
126a. LAZAROW, A. *Proc. Soc. Exper. Biol. and Med.* 66: 4, 1947.
127. LAZAROW, A. AND S. L. PALAY. *J. Lab. and Clin. Med.* 31: 1004, 1946.
128. LEECH, R. S. AND C. C. BAILEY. *J. Biol. Chem.* 157: 525, 1945.
129. LEHMANN, H. *Biochem. J.* 33: 1241, 1939.
130. LEVEY, S. AND B. SUTER. *Proc. Soc. Exper. Biol. and Med.* 63: 341, 1946.
131. LEWIS, L. A., J. MOSES AND R. W. SCHNEIDER. *Am. J. Med. Sci.* 213: 214, 1947.
132. LIEBEN, F. AND E. EDEL. *Biochem. Ztschr.* 244: 403, 1932.
133. LIEBIG, F. VON. *Liebig's Ann.* 121: 80, 1862.
134. LOWRY, P. T. AND D. M. HEGSTED. *J. Lab. and Clin. Med.* 30: 839, 1945.
135. LUKENS, F. D. W. AND F. C. DOHAN. *Endocrinology*, 30: 175, 1942.
136. MARTINEZ, C. *Rev. Soc. argent. biol.* 21: 332, 1945.
137. MARTINEZ, C. *Rev. Soc. argent. biol.* 21: 254, 1945.
138. MARTINEZ, C. *Rev. Soc. argent. biol.* 22: 135, 1946.
139. MARTINEZ, C. *Rev. Soc. argent. biol.* 22: 428, 1946.
140. MARTINEZ, C. AND O. ORIAS. *Rev. Soc. argent. biol.* 21: 290, 1945.
141. MENTEN, M. L. AND JANOUCH. *Proc. Soc. Exper. Biol. and Med.* 63: 33, 1946.
142. MILLER, H. C. *Endocrinology* 40: 251, 1947.
143. MIRSKY, I. A. *Proc. Soc. Exper. Biol. and Med.* 59: 35, 1945.
144. MITCHELL, H. S. *Proc. Soc. Exper. Biol. and Med.* 32: 971, 1934–5.
145. ORIAS, O. *Rev. Soc. argent. biol.* 20: 199, 1944.
146. PALAY, S. L. AND A. LAZAROW. *Anat. Rec.* 96: 55, 1946.
147. PENHOS, J. C. *Rev. Soc. argent. biol.* 22: 394, 1946.
148. PERALTA, R. B. *Rev. Inst. Salubridad y Enferm. Trop.* 6: 117, 1945.
149. PRETI, L. *Zeitschr. Physiol. Chem.* 62: 354, 1909.
150. RICHARDSON, G. M. AND R. K. CANNAN. *Biochem. J.* 23: 68, 1929.
151. RIDOUT, H. H, A. W. HAM AND G. A. WRENSHALL. *Science* 100: 57, 1944.
152. RUBEN, J. A. *Science* 101: 536, 1945.
153. RUBEN, J. A. AND K. YARDUMIAN. *Am. J. Clin. Path.* 15: 230, 1945.
154. RUBEN, J. A. AND K. YARDUMIAN. *Science* 103: 220, 1946.
155. RUBEN, J. A. AND K. YARDUMIAN. *Am. J. Clin. Path.* 16: 257, 1946.
156. SCHNEIDER, R. W., L. LEWIS, J. MOSES AND E. P. MCCULLAGH. *Proc. Central Soc. Clin. Res.* 17: 17, 1944.
157. SCOTT, C. C., P. N. HARRIS AND K. K. CHEN. *Endocrinology* 37: 201, 1945.
158. SEIDEN, G. *Anat. Rec.* 91: 187, 1945.
159. SHIPLEY, E. G. AND K. H. BEYER. *Endocrinology* 40: 154, 1947.
160. SHIPLEY, E. G. AND R. K. MEYER. *Am. J. Physiol.* 148: 185, 1947.
161. SHIPLEY, E. G. AND A. N. RANNEFELD. *Endocrinology* 37: 313, 1945.
162. SHIPLEY, E. G., A. N. RANNEFELD AND K. H. BEYER. *Federation Proc.* 4: 64, 1945.
163. STOLL, W. *Zeitschr. Naturforsch.* 1: 592, 1946 (*Chem. Abst.* 41: 5984, 1947).

164. THOMAS, T. B. AND G. A. EMERSON. *Proc. Texas Acad. Sci.* 28: 89, 1945.
165. THOROGOOD, E. *Federation Proc.* 3: 48, 1944.
166. THOROGOOD, E. AND B. ZIMMERMAN. *Endocrinology* 37: 191, 1945.
167. TIPSON, R. S. AND J. A. RUBEN. *Arch. Biochem.* 8: 1, 1945.
168. WALPOLE, A. L. AND J. R. M. INNES. *Brit. J. Pharmacol. and Chemotherapy* 1: 174, 1946.
169. WEBER, H. *Nature* 158: 627, 1946.
170. WEINGLASS, A. R., E. G. FRAME AND R. H. WILLIAMS. *Proc. Soc. Exper. Biol. and Med.* 58: 216, 1945.
171. WIELAND, H. AND F. BERGEL. *Ann. Chem.* 439: 196, 1924.
171a. WILDER, R. M. *Proc. Diabetes Assoc.* 6: 389, 1946.
172. WOEHLER, F. *Ann. Pharm.* 24: 241, 1838.

RENAL FUNCTION IN EARLY LIFE

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IT IS NOW GENERALLY RECOGNIZED that the function of an organ may alter as development proceeds. Physiologists were slow to appreciate this fact, but the work of the last 25 years has demonstrated its importance so clearly that several books have been written on the subject. In these Windle (128) and Barcroft (9) have dealt mainly with function during fetal life while Smith (110) has confined himself largely to the physiology of infancy. The time now seems to have come, however, to review certain aspects of these new developments in more detail. The infant kidney has been selected in the first instance because our knowledge of it has reached a stage at which a detailed and critical résumé of the established facts has become profitable and, in consequence, advisable. This article has been written, therefore, to compile knowledge which has already been acquired, to point out the gaps in that knowledge and finally to suggest various theories and lines of work which might profitably be pursued. The functional abnormalities of the kidney, which give rise to the Fanconi and other syndromes (113, 114), will not be discussed since it is felt that they are pathological entities and as such require rather different treatment. Since the basis of this review will be a comparison of the infant with the adult, some reference to adult function will be inevitable, but in general it has been assumed that the reader will possess a working knowledge of adult renal physiology.

GENERAL CONSIDERATIONS

Work of the Kidney and the Internal Environment of the Newborn

The function of the kidney may be defined in the most general terms as the regulation of the constancy of the internal environment. In utero this regulation is largely carried by the placenta but within a few minutes of birth the kidney must take up the task. From that time onward it must excrete all the nitrogenous end-products of the body. Under the partial direction of the post-pituitary body and the cortex of the suprarenal gland, moreover, the kidney must stabilize the osmotic pressure and the chemical composition of the circulating medium. For the rest of the animal's life the kidney must play a leading part in regulating the volume of the extracellular fluids. This is a matter of great concern to pediatricians and infant physiologists but the mechanism by which it is brought about is still very obscure. By excreting non-volatile acids, moreover, the kidney from birth will share with the lungs the task of maintaining the pH of the body within the narrow limits of normality. The efficiency with which all these operations are carried out by young animals forms the subject matter of this review. Any study of their renal function, therefore, should begin with an examination of the volume and composition of the internal environ-

ment and of the circulating fluid which bathes the renal cells. It is not possible in an article of this length to do so in any detail, but it is necessary to point out various ways in which these fluids in infants differ from those in adults. *a*) The serum of an infant contains less albumin and globulin that that of a healthy adult (53). Hence the colloidal osmotic pressure of the serum will be lower and this in turn will tend to favor a more rapid glomerular filtration rate and a more rapid outpouring of fluid into the tissue spaces. *b*) It is generally conceded that the osmotic pressure of the cells at all ages is the same as that of the extracellular fluids, i.e., of the internal environment; but whereas that of the former is mainly due to K salts, that of the latter is due to the Na salts of Cl and HCO_3. All published analyses have shown that newborn animals and fetuses contain more Na and Cl/kgm. of body weight than do adults of the same species and since newborn animals have the same concentration of Na in their sera as adults (18, 84) this has generally been taken to indicate that they contain more extracellular fluid/kgm. of body weight. This has been confirmed by a comparison of the volume of the body occupied by thiocyanate and radio-sodium in infants and adults. It is to be noted that the thiocyanate space was appreciably smaller than the sodium space at all ages (39). In other words, per unit of cell mass infants have a much larger volume of internal environment than adults have. Premature babies may exhibit this peculiarity in an extreme degree for they may be frankly edematous at birth or become so shortly afterwards. *c*) A number of authors have reported on the acid base balance of the serum of newborn infants. Seham (108) found plasma bicarbonate within normal limits, but Branning (16), Hoag and Kiser (54), Lippard and Marples (72), Lucas *et al* (73), Marples and Lippard (76, 77) and Ylppö (130, 131) have all reported values which were below those usually found in adults, and their work has been interpreted as meaning that many babies are in a state of acidosis for some little time after birth due to an accumulation of fixed acids. *d*) The concentration of inorganic P in the plasma of a baby tends to lie between 5 and 8 mgm/100 cc. (7, 34, 120). In adults the range for this ion is generally given as 2.5 to 4.5 mgm/100 cc. *e*) The serum K was found to average 28.1 mgm/100 cc. by Kotikoff (68) during the first month of human life, and 30.6 mgm/100 cc. by McCance and Young (84) in babies under 14 days old. An average figure of 21.9 was obtained by the latter authors for healthy adults. No one can set out to explain renal function in early life without taking cognizance of these differences between infants and adults.

Methods of Investigation and of Comparative Expression

The methods which have yielded so much information about the adult kidney are almost all available for the study of the infant kidney but some of them require adaptation to a more micro scale. Workers have not always checked their modifications for infant work against adult material, but any one who sets out to study renal function in young animals should be prepared to make similar investigations on adults by the same methods, because the basis of every study must be comparative. Some measurements, e.g., those of osmotic pressure, may be directly compared and sometimes the principle of an internal standard may be employed. Thus the NH_3/total N or the P/total N ratio in the urine of adults and infants may be com-

pared. Sometimes, however, a more arbitrary standard must be used. This is outstandingly so when clearances are to be compared. Surface area, body weight and kidney weight have all been suggested. It makes a large difference which basis or standard is employed, since the surface area of an adult is 8.25 times that of an infant at birth whereas the kidneys weigh about 13 times and the body 21.5 times as much. These and other possible standards of comparison, such as the basal metabolic requirements, and the sources of information were discussed by McCance and Young (84) and further data about weights of infants and their organs may be found in an article by Cruickshank and Miller (26). Of these standards, surface area would appear to be the best because per unit of surface area clearances of children over the age of 2 are of the same magnitude as those of adults (55, 86). Some investigators, however, have been dissatisfied with this basis of comparison for infants because by it clearances in infancy appear to be so low. This, however, is one of the important discoveries about the renal function of infants and to establish another basis for newborn children would only confuse the issue. All investigators, however, should state clearly the 'correction' factors which they have employed or give the surface area of their infant subjects. A much more subtle difficulty has been encountered by those people who have been administering substances to newborn and adult animals and comparing the effects on renal function. If the renal functions are to be compared on a basis of surface area, should these doses be calculated and equalized on a basis of surface area or of body weight? It is suggested that if renal functions are to be compared, the object of dosage should be to raise or to lower serum levels to the same degree at both ages and that the doses should be calculated on those terms. In practice this means dosing in terms of body weight or of extracellular fluid volume.

Species Differences

One of the contributions of Homer Smith and his associates to renal physiology was the attention which they gave to species differences. There is every reason to believe that these differences will be exaggerated in very young animals for no two species are born at the same stage of development. Investigators should be on the look out for these differences and should always state clearly both the age and the type of animal used.

RENAL FUNCTION BEFORE BIRTH

Until birth the regulation of the internal environment can be carried out unaided by the placenta because babies, normally developed in almost every way, may be born with functionless urinary tracts. The kidney, however, normally begins to function before birth, if only to a limited extent (52, 91). Even as early as the second or third month of fetal life, tubule cells of man growing in tissue culture have been observed to form small cysts and to secrete dyes into their lumina (21), and an analysis of liquor amnii at different stages of development has indicated that while the osmotic pressure of this fluid gradually falls, the concentration of urea and uric acid in it steadily rise (44). It is unfortunate that studies of the maternal and fetal bloods were not combined with those of the liquor, but the findings suggest that long before birth the kidney begins to excrete a urine of low specific gravity containing

urea and uric acid in appreciable amounts. They also suggest that the amniotic sac must be osmotically isolated from both mother and fetus for a large part of its existence. Butler (19) has recently shown that inulin injected into the mother may be detected in the urine of babies delivered within two hours, so that this substance must not only pass the placental membrane but be excreted by the fetal kidney. Wells (122, 123) delivered fetal rats into the abdominal cavity of the mother about 16 hours before term, and blocked the outlet of their bladders by cautery. He found that urine was formed and distended the urinary tracts above the obstruction, and that phenosulphonephthalein was excreted by these fetuses. The urine secreted by a group of 11 control animals averaged 2.25 mgm/grams fetus/hr. Into 6 animals he injected 75 mgm. of urea in a volume of 0.15 cc. and from these he collected an average of 4.5 mgm. of urine/grams of fetus/hr. He collected an average of 5.1 mgm. of urine/grams of fetus from 4 animals to whom he had given 0.15 cc. of distilled water and he concluded that fetal rats secreted urine more rapidly than was generally supposed during the last two days of uterine life. By employing a suitably sized animal and the appropriate technique it ought to be possible to work out the division of labor between the placenta and the kidney throughout a large part of fetal life.

Tausch (117) catheterized a large number of babies at birth and concluded that the urine of the last few hours or days of fetal life was generally acid and had a lower specific gravity than that found in adults. It contained creatinine and sometimes albumin, and the average non protein N amounted in that series to 0.155 per cent. Work recently carried out by McCance and von Finck (79) is in substantial agreement with these conclusions. Fetal urine characteristically contains very low concentrations of urea and other nitrogenous end-products and has a low osmotic pressure, whereas that of the mother passed at the same time may be highly concentrated. Since mother and fetus must be regarded as being in osmotic equilibrium, there is a problem to be solved here which might give us a clue to much of the renal physiology of the infant.

RENAL FUNCTION AFTER BIRTH

Urine Volumes and Osmotic Pressure

It may be assumed that a child is fully hydrated at birth, but in a state of nature it gets little fluid from the breast for at least 24 or often for 36 or even 48 hours. An article by Thomson (118) may be consulted for data about the fluid intake of newborn infants and an account of the volumes and the characteristics of the urine passed by them. In some hospitals newborn babies are given water, but in many they are not and in a few the infants are deliberately given no fluid for 36 hours or more even if they are to be reared from a bottle. Dehydration for this length of time would leave an adult in a state of considerable hydropenia and, although they do not sweat freely at this age, there is reason to believe that newborn babies may lose a greater proportion of their body water in the same time, for they have proportionally a larger surface area from which to evaporate moisture (124). Against this must be set Heller's (48) observations on newborn rats which showed that these animals had a surprisingly small extrarenal loss of water and the explanation of this fact probably lies in the findings of Levine and Marples (71). These workers demonstrated that the in-

sensible loss of water varied directly at all ages with the heat production—and this is well known to be low in newborn infants. Bailey and Murlin (6) found the metabolism of the newborn infant under basal conditions to be 25 Cals/sq.m/hr., whereas young adults have a basal heat production of about 40 Cals/sq.m/hour (35). It may be assumed with confidence, however, that babies are as a rule short of water from about the 4th to the 36th hour of their lives. Very little is known about the effect of this physiological hydropenia upon the chemistry of the internal environment and not much more about the way in which the kidney reacts to it, but much of this information should be easy to obtain. Our knowledge at present may be summarized as follows:

1) The blood urea rises between birth and the third day of life and with it in all probability the uric acid, NPN and other nitrogenous end-products (8, 73, 106, 107, 109). The rise in the blood urea may not be great and averaged 10.2 mg/100 cc. in a small series of 12 infants. Much higher figures have been observed in some premature babies (133). The concentration of urea in the blood begins to fall about the third day of life (for references, recent data and discussion see McCance and Widdowson, 82).

TABLE 1. CHANGES IN THE CONCENTRATION OF N AND P IN THE URINE DURING THE FIRST 10 DAYS OF LIFE

	AGE					
	0	5–24 hr.	24 hr.–4½ days	4½–6 days	6–7 days	7 days and over
Total N						
mgm/cc. of urine	0.70	5.45	5.60	1.92	3.22	2.11
Inorganic P						
μg/cc. of urine	6.16	116.0	106.0	41.1	79.0	65.0

The blood urea also rises for some days after birth in newborn rats (126). 2) The urine becomes more concentrated soon after birth and urates are frequently deposited in it as it cools. The output goes up later as the intake of milk increases. The figures in the table were obtained by McCance and von Finck (79) and show the general trend. It must be emphasized that these figures are averages and there was considerable variation from one child to another. All who have studied the urine at this age are in agreement about the reality and magnitude of these variations (118). Miller (87) has made similar observations on gastric juice, and it is evident that a number of points about the physiology of infancy can only be settled by a statistical approach. 3) No statement can be made about the volume of urine passed per minute before birth but the volumes of urine passed on the second and third days of life are small. In Thomson's series (118) they averaged only 20.6 cc/24 hrs., which is probably less than 100 cc/sq.m/24 hours, and one of the two babies with dehydration fever (119) passed less than 50 cc/sq.m/24 hr. for three consecutive days. Smith (111) made observations on premature and full-term babies who were given no water for nearly 48 hours and found that they were passing about 200 cc/sq.m/24 hrs. 4) The specific gravity of the urines passed at this time is of the order of 1012 to 1015 (118), and the osmotic pressure 450 m. osmols/l. (46).

Smith (111) found values of 400 to 600 m. osmols/l., and Thomson (119) one for *baby B* with dehydration fever which was probably not much higher. If the serum be taken to have an osmotic pressure of 300 m. osmols/l., it will be seen that the infant urines are generally more concentrated than the plasma on the second day of life, but after shorter periods of water deprivation healthy adults have been shown to produce urines with osmotic pressures of 1000 to 1300 m. osmols/l. so that it is clear that the human infant does not normally produce such hypertonic urines as an adult. When McCance and Young (84) first discussed this, their data had been derived from full-term babies who had been born for at least a week and who had never been deprived of water for very long. Their conclusions were based upon chemical analyses which probably gave results somewhat lower than the true U/P osmotic ratios. They concluded that the kidneys of infants consistently produced "hypotonic urines from plasmas from which adults would certainly have produced hypertonic urines at comparable urine volumes". The recent data do not conflict with their conclusions in any way. In discussing a series of premature babies, moreover, aged 2 to 7 days, Young, Hallum and McCance (133) pointed out that the U/P osmotic ratios as measured by them were one or more in a number of the children (the ratios would have been higher had they been based upon the actual freezing points). They thought that in some instances these high ratios were due to the high blood urea found in these babies and Aldridge (3) noted in older children that when the serum chlorides were within normal limits, little NaCl was excreted in the urine which was certainly hypotonic. After NaCl or normal saline had been given to these babies, however, the serum values became quite abnormal and the concentration of NaCl in the urine went up to 1.39 per cent. These urines must have had osmotic pressures of at least 600 or 700 m. osmols/l. It is possible that many babies do not begin to produce hypertonic urines until the urea or sodium salts in their plasmas have risen to abnormal heights and it is to be regretted that Thomson's (119) study of two babies with dehydration fever should not have included investigations of the serum chemistry. It may be concluded that babies aged 2 to 4 days are hydropenic, yet they produce urines with osmotic pressures which are sometimes no greater than that of the serum and never as great as that of the urine passed by a healthy adult after being deprived of water for a similar length of time. This work on man followed some very excellent studies on puppies which were made on the Continent of Europe between 1925 and 1936 by Kramár (66, 67), Schiff, Bayer and Choremis (104), Schiff (103), and particularly by Kerpel-Fronius (27, 59, 60, 61, 62, 63). These investigators were fully aware of the fact that young puppies could not concentrate their urine as could adult dogs and Kerpel-Fronius actually stated in 1932 that the same was true of all suckling mammals (89). A knowledge of this fact and of the well-known French dictum about the 'urine obligatoire' (4, 23) enabled these men to develop a method of dehydrating puppies by feeding them upon partially evaporated milk. They showed that *a*) the same diet did not dehydrate adult dogs and *b*) it was only possible to dehydrate the pups in this way if the diet contained a certain amount of protein. It was assumed that the inability of the kidney to concentrate led to a relative polyuria and this in turn led to the dehydration which was obvious clinically and measurable osmotically after four days. High praise must be given to these pioneer investigators.

It will be recalled that the volumes of urine passed by babies during the stage of physiological hydropenia were between 80 and 200 cc/sq.m/24 hrs. These volumes are surprisingly small. The two men dehydrated by Black *et al* (14) passed about 350 cc/sq.m/24 hrs., but their diets contained between 9 and 10 grams of NaCl/day and 10.4 to 13.8 grams of N. This quantity of urine, however, is little more than that excreted by Nadal, Pedersen and Maddock's *subject E.B.*, who excreted about 330 cc/sq.m/24 hrs. on a salt poor 'dry' diet. Gamble's (41) subject passed 290 cc/sq.m/24 hrs. and Ladell's men (69) somewhat less (about 230 cc/sq.m/24 hrs.), while the person who was given nothing to eat or drink by Nadal, Pedersen and Maddock (90) passed 440 cc. in the 24 hours which would be about 250 cc/sq.m. A similar figure was obtained by Winkler, Danowski, Elkinton and Peters (129). How are these findings to be reconciled with the views of Kerpel-Fronius? The human infants do not produce concentrated urines, yet they do not appear to have any polyuria. There are several possible explanations. The volume of the 'urine obligatoire' varies inversely as the concentrating capacity of the distal tubules and directly as the quantity of the osmotically active material reaching the distal tubules per minute. The latter can only be large if the glomerular filtration rates are high, and although nothing is known about the glomerular filtration rate of Kerpel-Fronius' pups, they were presumably relatively high. If the glomerular filtration rates of infants aged 2 days were sufficiently low, then the infants might have low minute volumes in spite of a poor concentrating power. The glomerular filtration rates of infants 2 days old are already known to be low (see later). They averaged 28 cc/sq.m/min. after giving fluids (31), but this is not low enough to explain the volumes of urine passed at this age by babies who are hydropenic, for if the volume of urine passed by a 2-day old infant be taken to be 150 cc/sq.m/24 hrs., this would be reduced to about 50 cc/sq.m/24 hrs. if the baby had the concentrating power of an adult. A glomerular filtration rate of 30 cc/sq.m/min., however, is little less than one half of the rate found in dehydrated adults by Black *et al* (14) and would lead one to expect urine volumes of the order of 450 cc/sq.m/24 hrs. in the infants. The volumes actually passed suggest that the glomerular filtration rates would have to have been of the order of 8 cc/sq.m/min. to explain the findings under discussion. The glomerular filtration rates, however, may well be as low as that in infants who have never received water or food. From all that is known about glomerular filtration rates this may be so, but no measurements have yet been made and, therefore, the relationship cannot be made quantitative. There is no information at present about the relative heights of the glomerular filtration rates in infancy and the reabsorptive capacity of the proximal tubules.

The Excretion of Water

After some controversial work in the two previous years (92, 101, 116, 124) it was shown by Lasch (70) that babies under 3 months of age excreted water less freely than babies over 3 months. This confirmed the findings of Aschenheim (5). This investigator had given babies 200 cc. of water at 6 a.m. after an overnight fast and collected their urine over the next 5 hours. In the second half year of life the infants excreted 200 cc. of water in this time, but less than this in the first half year and in the first three months only some 100 cc. Aschenheim considered that these results were

due to the fact that the tissues of the younger infants had a very high affinity for water. She did not attribute them to functional underdevelopment of the kidney. Lasch gave the babies under 3 months of age 100 cc. and those between 3 and 6 months 150 cc. of water, so that he employed more comparable tests. Unfortunately, Ohlmann (92) and Rominger (101) did not arrange their experiments in a similar way and little or no further work of a similar kind seems to have been done with human subjects. There is little doubt, however, that these results were correct, although they must now be extended and confirmed, for Adolph (1) has found that young puppies excrete water at a much slower rate than adult dogs and Heller (48) and McCance and Wilkinson (83) have found the same to be true of newborn rats. In fact, the administration of 5 per cent or even more of the body weight of water produces little if any diuresis in the newborn of this species.

The excretion of water cannot be discussed without reference to the post pituitary antidiuretic hormone. Heller (46) has published some important papers on this subject. He has shown that the administration of post pituitary hormone to infants does not lead to much concentration of the urine. The tests were made on babies over a week old who were passing the large volumes of dilute urine usual at that age. It is perhaps unfortunate that water was not given to the infants, since the action of the post pituitary hormone can be demonstrated with most certainty when the animal is carrying a considerable water load. Be that as it may, Heller (49) has also found that the pituitaries of young animals can only be made to yield a fraction of the active material per 100 grams of body weight obtainable from the pars nervosa of adult glands. It is certainly an interesting adaptation of nature that for the time after birth during which the kidney appears to produce little or no diuresis after the administration of water and to be at the same time unresponsive to post pituitary hormone, the gland should contain and presumably manufacture so little.

Glomerular Filtration Rate

It is recognized that the glomerular filtration rate in adults averages about 70 cc/sq.m/min. and that it is relatively unaffected by the hydration of the subject (14), at any rate, so long as he continues to eat. Barnett (10), who employed an indirect method to estimate the inulin clearances of some newborn infants, found that on the basis of surface area the inulin clearances at birth were less than 50 per cent of those in adult life. Young and McCance (134) showed in a few babies that the inulin clearances, i.e., the glomerular filtration rates, were only of the order of 30 cc/sq.m/min. and the recent work has in general gone to confirm these findings and to show that, while the rates may be very variable from one baby to another, the average figures rise with age to approach the adult levels by the end of the first year or 18 months of life. Thus Dean and McCance (31) found the glomerular filtration rates averaged 28 cc/sq.m/min. in babies a few days old. West, Smith and Chasis (125) and Rubin, Bruck and Rapoport (102) have found the mannitol clearances in very young infants to be much lower than those of adults. The former authors considered that adult levels were reached as early as the second month but the latter not till the second or third year. Somewhere between the two will probably turn out to be true for the *average* baby, but large individual variations must be expected.

After examining a small series of infants McCance and Young (84) considered

that the glomerular filtration rates varied with the hydration of the baby. Very similar results were obtained quite independently by Barnett (10) who made an elegant study of a baby with extravertion of the bladder (12). Both ureters were catheterized and the kidneys compared. This dependence of the glomerular filtration rate upon the hydration of the animal is not peculiar to the human infant. It is shown by the adult rabbit (56, 127) and by other species. It may (40) or may not (47) be true of the rat. Recent work in the United States, however, has not demonstrated any close relationship between the volume of the urine and the glomerular filtration rate (11), but some of this work was done with mannitol as the index of glomerular filtration and this substance acts as an osmotic diuretic, so that few really low minute volumes were at first obtained. Barnett *et al* (13) have now reverted to inulin as the index of glomerular filtration rate and shown that by varying the water intake of premature infants the inulin U/P ratios could be made to vary from 3.9 to 127. The latter figure indicates that the glomerular filtration rates must have remained high even when the urine volumes had fallen to low levels. Babies have still to be tested by the new techniques under conditions of real dehydration and this should be done from the clinical point of view for, if it were to be established that the glomerular filtration rates fall to low levels in really dehydrated infants, then it would be easy to explain the grossly abnormal serum chemistry often found in them.

Urea Clearances

Urea clearances have been the standard tests of an adult's renal efficiency for a number of years, and a great deal is known about them. At urine volumes over 2 cc/min. the clearances are at a maximum value which is about 72 cc/min. As the urine volumes fall the clearances also fall and at 1 cc/min. average 54 cc. At urine volumes of 0.3 cc/min. the clearances are of the order of 34 cc. McCance and Young (84) determined the urea clearances of healthy babies of various ages and found that in the first 14 days of life they were far below those of adults when the two were compared on the basis of surface area. These findings might possibly have been predicted from some work of Stransky and Bálint (115) on Ambard's coefficient in infancy, or from what has already been said about the glomerular filtration rates and been confirmed by all subsequent investigators (102, 125).

McCance and Young (84) found, as might have been expected, that on the whole the urea clearances in infants varied as they did in adults with the degree of hydration of the subject, but Gordon, Harrison and McNamara (42), who have also determined the urea clearances in the early days of life and agree about their being so much lower than those of adults, did not find that they varied with the minute volumes of the urine. The original findings have, however, been confirmed by Barnett *et al* (13) and by Dean and McCance (unpublished data). Young and McCance (134) and Gordon *et al* (42) found that the urea clearances rose towards the adult levels as the babies grew, and Young and McCance (134) showed that they were approaching adult levels towards the end of the first year of life (102).

TUBULAR FUNCTION

Creatinine Clearances

In animals other than man and the higher apes the exogenous creatinine clearances are equal to the inulin clearances (112). In adult human beings the exogenous

creatinine clearances are greater than the inulin clearances. The ratio of the one to the other is of the order of 1.3 and some creatinine is generally considered to be excreted by the tubules. The endogenous creatinine clearances are said to be equal to the inulin clearances but, since some of the chromogenic material is most probably not creatinine, it is impossible to say what scientific value should be assigned to these endogenous creatinine clearances. Brod (17) has examined the endogenous creatinine/mannitol clearance ratios in infancy and found them less than unity and Dean and McCance (31) studied the exogenous creatinine/inulin clearance ratios in babies a few days old and found them to average 1. Both these results suggests that at birth the tubule cells have not yet developed the property of excreting creatinine which they will acquire in later life.

Diodone (B.P.) and p-Aminohippuric Acid Clearances

These substances are excreted to some extent by glomerular filtration but they are also actively excreted by the tubules. Their clearances have been used to measure the blood flow through the kidney by virtue of the fact that if the quantity of one or the other of them in the plasma is small, the whole of it is removed in one passage through the kidney. The maximum amount which the tubules can excrete from high plasma levels (Diodone or p-aminohippuric acid Tm) has been used as a measure of the active tubular mass. Dean and McCance (31) measured the diodone clearances from low plasma concentrations in the first four days of life and found them to be low by adult standards on the usual surface area basis. They obtained an average value of 66 cc/1.73 sq.m/min. against one of 600 cc/min. for adults. They also found the diodone/inulin clearance ratios to be low, which suggested to these authors that the diodone was being excreted much less readily by the tubules in infancy than in adult life, and that its clearance could not be used to measure the blood flow through the kidney at this age. The inulin/diodone clearance ratios, or the so called filtration fractions of Dean and McCance, averaged 0.42 and those of Barnett *et al* (13) 0.34 in 4 premature infants under 14 days of life. The average for adults is much lower. Workers in Homer Smith's Department (125) and also Rubin, Bruck and Rapoport (102) have studied the clearances and the Tm values of p-aminohippuric acid in older babies and communicated their results to the Fifth International Congress of Pediatrics. At the earlier ages they also found low clearances and Tm values for p-aminohippuric acid; as the infants grew the clearances and Tm values rose and approached adult levels towards the end of the first year.

Mineral Clearances

Very much less is known about mineral than about urea, inulin or creatinine clearances and no adult standards have been laid down for potassium, sodium or chloride. The clearance of a true non-threshold substance such as inulin should only be affected by the glomerular filtration rate and perhaps by the minute volume, but the clearance of a threshold body such as glucose or chloride depends not only upon the glomerular filtration rate and the minute volume, but also upon the concentration in the serum and the reabsorptive activity of the tubules. This last is partly controlled by the suprarenal cortex—at any rate so far as sodium, chloride and potassium are concerned—and it is not possible to make any measurement of the extent of this.

Variations in suprarenal activity, however, are probably responsible for some of the puzzling facts about mineral clearances. The rate of excretion of NaCl, for instance, often appears to depend upon intake rather than serum level, and Borst (15) has recorded variations in chloride output which are the very reverse of those which a knowledge of serum values would have led one to expect. McCance and Young (84) determined the sodium, chloride and potassium clearances in a number of newborn full-term babies and they showed that the clearances were much lower than those of adults. On the usual basis of surface area the figures for infants tended to be about one-fifth of those of the adults tested at the same time. It has already been pointed out that the concentrations of these elements in the sera of infants are fully as high as or higher than in those of adults, so that the cause of the low clearances must lie in the kidney itself or in the ductless glands which control the excretion of these ions by the kidney. The low clearances indeed may be the reason for the high values found in the blood and the large volume of extracellular fluids at this age. They may also be responsible for much of the edema which affects premature infants. Young, Hallum and McCance (133) found that the Cl clearances were much lower in premature than in full-term infants. At all ages the Cl clearances tended to fall as the volumes of urine passed per minute decreased. Young and McCance (134) found that the K clearances also varied with the minute volume. It is still not known whether the low glomerular filtration rates will fully explain these low clearances or whether the tubules reabsorb more of the minerals from the glomerular filtrate in infancy than they do in adult life. A further analysis can only be made when the glomerular filtration rate and the sodium and chloride clearances have been determined simultaneously.

Some small amount of attention has been given to the rate of excretion of P and its clearances in adults. Phosphate clearances have been found by several people (33, 93) to be of the order of 10 to 11 cc/sq.m/min., but there are many curious features about them which are so far unexplained. They have been found to be lower in the forenoon than at other times of the day and night (38, 65). Dean and McCance (33) found the phosphate clearances of infants less than 14 days old to be greater when the minute volumes were large than when they were small and to range from 0.12 to 3.7 cc/sq.m/min. This range is far below the average for adults and it is evident that these clearances resemble all others so far investigated in being very much lower in infancy than they are later in life. It is not known at what age the phosphate clearances approach the adult levels, but one hydrocephalic child aged 5 months investigated by Dean and McCance had a phosphate clearance of 7.5 cc/sq.m/min., which is within the adult range. It is to be noted that these low clearances may be the explanation of the high serum phosphorus characteristic of infants and young children, but until simultaneous glomerular filtration rates and phosphate clearances have been carried out on young infants it will not be possible to state whether the glomerular filtration rates are low enough to account for the clearances, or whether exaggerated tubular reabsorption also plays a part.

Osmotic Diuresis

If a strongly hypertonic solution of urea or sodium chloride is administered to a hydropenic adult or to a full-grown rat which has been deprived of water for 12 hours,

a very characteristic response is evoked. This response has been investigated by McCance and Young (85); Chambers *et al* (22); McCance (78); Hervey, McCance and Tayler (50, 51), and by McCance and Wilkinson (83). It consists of *a*) a diuresis which may be very intense for a time but always subsides before all the NaCl has been excreted and *b*) a fall in the osmotic pressure of the urine in spite of the hypertonic state of the animal's body fluids. Hervey, McCance and Tayler attributed this fall in osmotic pressure to the fact that the distal tubules were only capable of carrying out a limited amount of osmotic work per minute so that when excessive quantities of osmotically active material were passed on to them by the proximal tubules as an isosmotic solution, they were unable to remove enough water to raise the osmotic pressure of the large volume of fluid to the height usual in dehydration. The response of a newborn animal to similar treatment differs considerably from that of an adult of the same species and newborn rats which were investigated by McCance and Wilkinson show these differences to a much greater degree than human infants. When 5 per cent of the body weight of 10 per cent NaCl or 20 per cent urea was administered to newborn rats by stomach tube, the young animals passed very little additional urine, although the changes in the serum chemistry were as great as or greater than in the adults. The urine of these newborn animals, moreover, which has always got a much lower osmotic pressure than the urine of the hydropenic adult, did not become more dilute but tended to become more concentrated. The absence of any appreciable diuresis means that the newborn animal is incapable of doing much if anything to restore the internal environment to its normal composition. Dean and McCance (32) have investigated the responses of newborn infants to similar treatment. In them a diuresis of small magnitude is obtained and the osmotic pressure of the urine may rise slightly or remain unchanged. The percentage of the dose of NaCl which is excreted is always much less than that excreted by an adult. There is no doubt that these effects and phenomena must be considered in connection with the low sodium and chloride clearances which have already been described and with the work of Aldridge (2, 3), Maizels and McArthur (75), Cooper (25) and others who have so often emphasized the rapidity with which an infant's serum chemistry may become highly abnormal. There is a great deal of quantitative work to be done before the relationships of all these phenomena to each other can be satisfactorily explained.

Acid-Base Regulation

This is a most important function of the kidney but it is a very difficult one to investigate quantitatively. The evidence that babies may remain in a state of acidosis for some time after birth has already been given. A change from breast milk to cow's milk has been said to alter materially the acid-base balance of the serum towards the acid side (72). The reactions of infant urines, however, have not been found to be highly acid. The average pH was close to that of a large group of adults. There are, however, interesting differences between the manner and the facility with which adults and infants excrete acid (79). Infants tend to have higher ratios of ammonia/acidity titrated to the phenolphthalein end point and also higher ammonia coefficients than adults. Both these findings suggest either that their kidneys are

coping with an acidosis or that there are few of the normal adult buffer substances in an infant's urine. It has been known for a very long time (105) that the urine of an infant may contain extraordinarily little inorganic phosphate. This was fully confirmed by McCance and von Finck, who showed that the P/N ratio in infancy was far lower than in adults and that in infancy much less of the titratable acidity was due to acid phosphates, and much more of it to organic acids than later in life. This small excretion of phosphates by newborn infants clearly makes it difficult for them to cope with an acidosis should one arise (96, 97, 98) and may be the explanation of the findings in the serum. The low phosphate clearances are also a part of this story. No one has yet compared the capacity of the tubules to produce ammonia in infancy and in adult life by creating an internal acidosis. This would be an instructive experiment especially in the light of the data which has just been discussed.

Cellular Metabolism

Differences in cellular metabolism must underlie the changes in renal function which accompany development, but so far little work has been carried out on this subject and there is no immediate prospect of being able to correlate metabolism with function. Kay (57, 58) reported that the phosphatase activity of embryonic rabbits' kidneys was low and that it remained below the adult levels for some days after birth. Epps (37) found that the cortex and medulla of the kidney of the newborn child were deficient in amine oxidase, as compared with the same parts of an adult's kidney. The activity of the oxidase had reached the adult level by the time the infant had reached the age of 3 months. Cutting and McCance (28, 29) showed that the oxygen consumption of the kidney cells of newborn rats was a little lower than that of adult cells when the slices were first set up. The oxygen consumption of the kidney cells of newborn pigs, cats and humans was higher than that of adults, but in all species the oxygen consumption of the newborn cells was better maintained. The addition of lactate or succinate to the slices of newborn kidneys raised the oxygen consumption much less than a corresponding addition to slices from adult kidneys. Another difference between the behavior of these slices at the two ages was that the adult slices disintegrated much more rapidly so that far more of the tissue N escaped into the surrounding medium. It was shown in rats that this disintegration could be prevented to a large extent by adding calcium to the medium and this addition made the adult slices behave in other ways more like those of newborn animals. The addition of calcium was without effect upon the slices of newborn animals (30).

Anatomy

Parts of the human kidney are still undeveloped at birth. Many years ago Eckhardt (36) stated that the glomeruli at birth were smaller and much more close together than they were in adults, but he considered that no new glomeruli were formed after full term. Potter and Thierstein (99) found that developing glomeruli could no longer be found after an infant had reached a weight of 2100 to 2500 grams. Thus many premature infants must possess this functional handicap whereas full-term infants should not. Evidence has already been quoted to show that premature infants have lower clearances of urea and salt than full-term babies, but to what

extent this is due to a numerical deficiency of glomeruli (and presumably therefore of nephrons) has not yet been made clear. According to Peter (95) the glomeruli are all formed, but the nephrons attached to those near the capsule are still very primitive and the loops of Henle are very short. The most central nephrons are in a much more advanced stage of development, but even they have many primitive features (88). If the loops of Henle are really the parts of the nephron responsible for the production of a hypertonic urine, these observations might be held to explain the inability of an infant to produce a concentrated urine, but unfortunately any theories about function based upon measurements of the loops of Henle are complicated by the fact that even in adults the loops are of very different length (94).

In some young animals the kidneys seem to be less well developed at birth than they are in man, for Riedel (100) found that in mice and in other animals which were born blind new glomeruli continued to be formed for some time after birth. Yoffey (132) has studied the elimination of trypan blue by the cells in the proximal tubules of growing rats. In newborn animals the nephrons in the peripheral part of the cortex were still undeveloped and the cells took up no dye. The proximal tubules in the deeper layers of the kidney took up dye and the nearer they lay to the medulla the more actively they did so. The ability of the cells to excrete dye was accompanied by the development of a brush border. Some attempt should be made to correlate these histological findings with any changes in enzyme activity which are known to take place at about the same time. The bearing of the histological data upon the function of urine formation, as revealed by studies of the whole animals, will be more difficult to interpret.

Trueta *et al* (121) have recently made some interesting discoveries about the renal circulation. They have confirmed that in the neonatal child the cortex makes up a much smaller proportion of the kidney than it does in later life, and consequently that the medullary and juxtamedullary glomeruli must have a particular significance at that time. They believe that these glomeruli have a blood supply which differs in important respects from those in the cortex. It is possible that the function of these inner nephrons differs from those lying nearer the cortex of the kidney and that something of this kind may underlie the functional differences between the kidneys of newborn and adult animals, but this is a matter of conjecture at present. Trueta *et al* have also demonstrated the development in certain circumstances of large arterio-venous shunts which may exclude most of the cortex from active circulation. These observations may go far towards explaining some of the functional abnormalities of the kidney with which physicians have been familiar for many years (20, 74, 80, 81), but it is difficult to see at present how their discoveries further the study of function in the newborn kidney. Of more importance would seem to be the histological data about the state of the glomerular epithelium. In adult life the glomerular tuft is covered with the thinnest of pavement epithelia so that there is a minimum barrier to filtration. In fetal life, however, the tuft is covered with a layer of tall columnar cells, which must be an effective barrier to ultrafiltration. These cells were described and illustrated long ago by Klein *et al* (64), but they have been redescribed quite recently by Grunewald and Popper (43). At full term the cells are much less conspicuous and are cubical rather than columnar and may not completely

cover the tuft (24). Coincidental with this developmental change in the shape of the glomerular cells there appears also to be one in their staining reactions with silver salts, for as the cells flatten they acquire a fine reticulum of argyrophil fibrils (45). There is thus an anatomical basis for the low glomerular filtration rates and low clearances of early life. In the first place the cortical glomeruli and nephrons may not yet have commenced to function. In the second place the glomeruli, even if functioning, are still partially covered by a layer of cells thick enough to make filtration a relatively slow process.

CONCLUSIONS

In this review a short description has been given of the anatomy, cellular chemistry and physiology of the infant's kidney. It has been shown to differ in all these respects from the adult organ, but it is not yet possible to correlate these differences to any extent or to discuss the functional immaturity of the newborn organ in terms of development, anatomy and cellular metabolism. In the future no doubt it will be possible to do this.

If it is permissible to generalize about the functional differences, it may be said that the kidneys of animals begin to form and to secrete a fluid which resembles urine before birth. They continue to do so after birth and there is probably no great functional development at that time. In the early days or weeks of life the newborn kidney is, however, a less effective organ than it will later become. Thus the glomerular filtration rates are lower and the clearances of all those substances which are excreted mainly by glomerular filtration are necessarily also lower. The tubules have not yet acquired to the full their capacity to excrete creatinine, diodone or p-aminohippuric acid, but their ability to reabsorb sodium, chloride and phosphate ions may develop more quickly than the glomerular filtration rate so that a very high proportion of these ions may be reabsorbed from the glomerular filtrates. Functionally, moreover, the kidney of the newborn animal has little flexibility. It excretes water, sodium chloride and urea, but if the mutual relationships of these in the internal environment are disturbed, the kidney makes a relatively feeble attempt to restore the status quo and this is true even when the disturbance has been very great. There is some slight evidence also that the same may be true of pH.

In the light of these results a study of the capacity and limitations of the kidney in the early days of life seems to be an important subject for research. If all is normal, the kidney of a baby will do the work required of it—Nature has seen to that—but if the child has become dehydrated, acidosed, or otherwise abnormal, or has to undergo some surgical operation, then the duty of the pediatrician must be to assist the kidney to restore and maintain the normality of the internal environment and he requires for this purpose all the knowledge with which the investigator can supply him.

REFERENCES

1. ADOLPH, E. F. *Physiological Regulations.* Lancaster, Pa.: Jaques Cattell Press, 1943. Pp. 266 et seq.
2. ALDRIDGE, A. G. V. *Arch. Dis. Child.* 16: 81, 1941.
3. ALDRIDGE, A. G. V. *Arch. Dis. Child.* 16: 182, 1941.

4. AMBARD, L. AND E. PAPIN. *Arch. internat. de Physiol.* 8: 437, 1909.
5. ASCHENHEIM, E. *Ztschr. Kinderheilk.* 24: 281, 1919.
6. BAILEY, H. C. AND J. R. MURLIN. *Am. J. Obstet. Gynec.* 71: 526, 1915.
7. BAKWIN, H. *J. Pediat.* 14: 1, 1939.
8. BÁLINT, A. AND E. STRANSKY. *Jahrb. Kinderheilk* 93: 3e folge; 43: 210, 1920.
9. BARCROFT, J. Researches on Prenatal Life. Blackwell Scientific Publications. Oxford: Mowbray & Co., 1946.
10. BARNETT, H. L. *Proc. Soc. Exper. Biol. and Med.* 44: 654, 1940.
11. BARNETT, H. L. Private communication. 1947.
12. BARNETT, H. L., A. M. PERLEY AND H. G. MCGINNIS. *Proc. Soc. Exper. Biol. and Med.* 49: 90, 1942.
13. BARNETT, H. L., H. MCNAMARA, R. S. HARE AND K. HARE. *Proc. Soc. Exper. Biol. and Med.* March 1948.
14. BLACK, D. A. K., R. A. MCCANCE AND W. F. YOUNG. *Nature* 150: 461, 1942.
15. BORST, J. G. G. *The Restoration of the Circulation after Gross Haemorrhage into the Intestinal Tract.* Lecture to the Dutch Gastroenterological Society. Oct. 27, 1940.
16. BRANNING, W. S. *J. Clin. Investigation* 21: 101, 1942.
17. BROD, I. Private communication. 1947.
18. BRUCH, H. AND D. J. MCCUNE. *Am. J. Dis. Child.* 52: 863, 1936.
19. BUTLER, B. C. Private communication. 1947.
20. BYWATERS, E. G. L. AND D. BEALL. *Brit. Med. J.* 1: 427, 1941.
21. CAMERON, G. AND R. CHAMBERS. *Am. J. Physiol.* 123: 482, 1938.
22. CHAMBERS, G. H., E. V. MELVILLE, R. A. HARE AND K. HARE. *Am. J. Physiol.* 144: 311, 1945.
23. CHAUSSIN, J. *J. Physiol. Path. gen.* 18: 895, 1920.
24. CLARA, M. *Z. Mikrosk.-anat. Forsch.* 40: 147, 1936.
25. COOPER, E. D. *Arch. Dis. Child.* 12: 359, 1937.
26. CRUICKSHANK, J. N. AND M. J. MILLER. *Medical Research Council Sp. Rep. Ser.* 86: 33, 1924.
27. CSAPO, J. AND E. KERPEL-FRONIUS. *Monatschr. Kinderheilk.* 58: 1, 1933.
28. CUTTING, M. AND R. A. MCCANCE. *J. Physiol.* 104: 288, 1946.
29. CUTTING, M. AND R. A. MCCANCE. *J. Physiol.* 105: 205, 1946.
30. CUTTING, M. AND R. A. MCCANCE. *J. Physiol.* 106: 405, 1947.
31. DEAN, R. F. A. AND R. A. MCCANCE. *J. Physiol.* 106: 431, 1947.
32. DEAN, R. F. A. AND R. A. MCCANCE. *Nature* 160: 904, 1947.
33. DEAN, R. F. A. AND R. A. MCCANCE. *J. Physiol.* 1948.
34. DENZER, B. S., M. REINER AND S. B. WEINER. *Am. J. Dis. Child.* 57: 809, 1939.
35. DE BOIS, E. F. *Basal Metabolism in Health and Disease.* London: Bailliere, Tindall and Cox, 1936.
36. ECKHARDT, C. T. *Virchows Arch.* 114: 217, 1888.
37. EPPS, H. M. R. *Biochem. J.* 39: 37, 1945.
38. FISKE, C. H. *J. Biol. Chem.* 49: 171, 1921.
39. FELLERS, F. X., H. L. BARNETT, H. MCNAMARA AND K. HARE. *Proc. Soc. Exper. Biol. and Med.* March 1948.
40. FRIEDMAN, M. *Am. J. Physiol.* 148: 387, 1947.
41. GAMBLE, J. L. *Proc. Am. Philosophic. Soc.* 88: 151, 1944.
42. GORDON, H. H., H. E. HARRISON AND H. MCNAMARA. *J. Clin. Investigation* 21: 499, 1942.
43. GRUENWALD, P. AND H. POPPER. *J. Urol.* 43: 452, 1940.
44. GUTHMANN, H. AND W. MAY. *Arch. Gynäk.* 141: 450, 1930.
45. HARE, K., L. UNGEWITTER, H. L. BARNETT AND H. MCNAMARA. *Proc. Soc. Exper. Biol. and Med.* March 1948.
46. HELLER, H. *J. Physiol.* 102: 429, 1944.
47. HELLER, H. *J. Physiol.* 103: 449, 1945.
48. HELLER, H. *J. Physiol.* 106: 245, 1947.
49. HELLER, H. *J. Physiol.* 106: 28, 1947.
50. HERVEY, G. R., R. A. MCCANCE AND R. G. O. TAYLER. *Nature* 157: 338, 1946.

51. HERVEY, G. R., R. A. MCCANCE AND R. G. O. TAYLER. *J. Physiol.* 104: 43P, 1946.
52. HEWER, E. F. *Quart. J. Exper. Physiol.* 14: 49, 1924.
53. HICKMANS, E. M., E. FINCH AND E. TONKS. *Arch. Dis. Child.* 18: 96, 1943.
54. HOAG, L. A. AND W. H. KISER. *Am. J. Dis. Child.* 41: 1054, 1931.
55. HOLTEN, C. *Acta. paediat.* 12: 251, 1931.
56. KAPLAN, B. J. AND H. W. SMITH. *Am. J. Physiol.* 113: 354, 1935.
57. KAY, H. D. *Biochem. J.* 20: 791, 1926.
58. KAY, H. D. *Brit. J. Exper. Path.* 7: 177, 1926.
59. KERPEL-FRONIUS, E. *Ztschr. ges. expt. Med.* 85: 235, 1932.
60. KERPEL-FRONIUS, E. *Monatschr. Kinderheilk.* 51: 400, 1932.
61. KERPEL-FRONIUS, E. *Ztschr. ges. expt. Med.* 90: 676, 1933.
62. KERPEL-FRONIUS, E. *Z. Kinderheilk.* 57: 489, 1935.
63. KERPEL-FRONIUS, E. AND F. LEOVEY. *Arch. Kinderheilk.* 94: 9, 1931.
64. KLEIN, E., J. BURDON-SANDERSON, M. FOSTER AND T. L. BRUNTON. Handbook for the Physiological Laboratory. *1*: text, *2*: plates. London: Churchill, 1873.
65. KLEITMAN, N. *Am. J. Physiol.* 74: 225, 1925.
66. KRAMÄR, E. *Jahrb. Kinderheilk.* 114: 356, 1926.
67. KRAMÄR, E. *Jahrb. Kinderheilk.* 115: 289, 1927.
68. KOTIKOFF, J. A. *Jahrb. Kinderheilk.* 138: 280, 1933.
69. LADELL, W. S. S. *Lancet.* 2: 441, 1943.
70. LASCH, W. *Z. Kinderheilk.* 36: 42, 1923.
71. LEVINE, S. Z. AND E. MARPLES. *Am. J. Dis. Child.* 40: 269, 1930.
72. LIPPARD, V. W. AND E. MARPLES. *Am. J. Dis. Child.* 46: 495, 1933.
73. LUCAS, W. P., B. F. DEARING, H. R. HOOBLER, A. COX, M. R. JONES AND F. S. SMYTH. *Am. J. Dis. Child.* 22: 525, 1921.
74. MAEGRAITH, B. G., R. E. HAVARD AND D. S. PARSONS. *Lancet.* 2: 293, 1945.
75. MAIZELS, M. AND C. B. MCARTHUR. *Quart. J. Med.* 22: 581, 1928–29.
76. MARPLES, E. AND V. W. LIPPARD. *Am. J. Dis. Child.* 44: 31, 1932.
77. MARPLES, E. AND V. W. LIPPARD. *Am. J. Dis. Child.* 45: 294, 1933.
78. MCCANCE, R. A. *J. Physiol.* 104: 196, 1945.
79. MCCANCE, R. A. AND M. A. VON FINCK. *Arch. Dis. Child.* 22: 200, 1947.
80. MCCANCE, R. A. AND R. D. LAWERENCE *Quart. J. Med. N. S.* 4: 53, 1935.
81. MCCANCE, R. A. AND E. M. WIDDOWSON. *J. Physiol.* 95: 36, 1939.
82. MCCANCE, R. A. AND E. M. WIDDOWSON. *Lancet.* 1: 787, 1947.
83. MCCANCE, R. A. AND E. WILKINSON. *J. Physiol.* 106: 256, 1947.
84. MCCANCE, R. A. AND W. F. YOUNG. *J. Physiol.* 99: 265, 1941.
85. MCCANCE, R. A. AND W. F. YOUNG. *J. Physiol.* 102: 415, 1944.
86. MCINTOSH, J. F., E. MÖLLER AND D. D. VAN SLYKE. *J. Clin. Investigation.* 6: 467, 1928.
87. MILLER, R. A. *Arch. Dis. Child.* 16: 22, 1941.
88. VON MÖLLENDORF, W. *Handbuch der mikroskopischen Anatomie des Menschen Harn und Geschlechtsapparat* (Erster teil). Berlin: Springer. p. 251.
89. VON MORITZ, D. AND E. KERPEL-FRONIUS. *Monatschr. Kinderheilk.* 54: 371, 1932.
90. NADAL, J. W., S. PEDERSEN AND W. G. MADDOCK. *J. Clin. Investigation.* 20: 691, 1941.
91. NEEDHAM, J. Chemical Embryology. Cambridge: Univ. Press., 1931.
92. OHLMANN, J. *Z. Kinderheilk.* 26: 291, 1920.
93. OLLAYOS, R. W. AND A. W. WINKLER. *J. Clin Investigation.* 22: 147, 1943.
94. PETER, K. Untersuchungen über Bau und Entwicklung der Niere (Erstes Heft). Gustav Fischer, Jena, 1909.
95. PETER, K. Untersuchungen über Bau und Entwicklung der Niere. (Zweites Heft). Gustav Fischer, Jena, 1927.
96. PITTS, R. F. *Science* 102: 49, 1945.
97. PITTS, R. F. *Science* 102: 81, 1945.
98. PITTS, R. F. *Ann. Rev. Physiol.* 8: 199, 1946.
99. POTTER, E. L. AND S. T. THIERSTEIN. *J. Pediat.* 22: 695, 1943.

100. RIEDEL, B. Entwicklung der Säugetierniere. Untersuch. anat. Inst. Rostock, 1874 (quoted by von Möllendorf q.v., p. 24).
101. ROMINGER, E. *Z. Kinderheilk.* 26: 23, 1920.
102. RUBIN, M. I., E. BRUCK AND M. RAPOPORT. Studies of the Maturation of Renal Function in Normal Children and the Renal Functional Disturbances During Various Forms of Kidney Disease in Childhood. Communication to the Fifth International Congress of Pediatrics. New York, 1947.
103. SCHIFF, E. *Ergebn. Inner. Med. Kinderheilk.* 35: 519, 1929.
104. SCHIFF, E., W. BAYER AND K. CHOREMIS. *Jahrb. Kinderheilk.* 109: 287, 1925.
105. SCHLOSS, O. M. AND J. L. CRAWFORD. *Am. J. Dis. Child.* 1: 203, 1911.
106. SCHLUTZ, F. W. AND K. J. V. PETTIBONE. *Am. J. Dis. Child.* 10: 206, 1915.
107. SEDGWICK, J. P. AND M. R. ZIEGLER. *Am. J. Dis. Child.* 19: 429, 1920.
108. SEHAM, M. *Am. J. Dis. Child.* 18: 42, 1919.
109. SHERMAN, D. H., G. W. PUCHER AND H. R. LOHNES. *Am. J. Dis. Child.* 10: 206, 1925.
110. SMITH, C. A. The Physiology of the Newborn Infant. Springfield, Illinois: Charles C. Thomas, 1945.
111. SMITH, C. A. Renal Function in Early Post-Natal Life in Premature and Mature Infants. Communication to the Fifth International Congress of Pediatrics. New York, 1947.
112. SMITH, H. W. The Physiology of the Kidney. New York: Oxford Univ. Press., 1937.
113. SNELLING, C. E. *J. Pediat.* 22: 559, 1943.
114. STOWERS, J. M. AND C. E. DENT. *Quart. J. Med. N.S.* 16: 275, 1947.
115. STRANSKY, E. AND BÁLINT. *Jahrb. Kinderheilk.* 93: 3ᵉfolge, 43: 350, 1920.
116. STRANSKY, E. AND O. WEBER. *Jahrb. Kinderheilk.* 93: 3ᵉfolge, 43: 368, 1920.
117. TAUSCH, M. *Arch. Gynäk.* 162: 217, 1936.
118. THOMSON, J. *Arch. Dis. Child.* 19: 169, 1944.
119. THOMSON, J. *Arch. Dis. Child.* 22: 200, 1947.
120. TODD, W. R., E. G. CHUINARD AND M. T. WOOD. *Am. J. Dis. Child.* 57: 1278, 1939.
121. TRUETA, J., A. E. BARCLAY, P. M. DANIEL, K. J. FRANKLIN AND M. M. L. PRICHARD. Studies of the Renal Circulation. Oxford: Blackwell Scientific Publications, 1947.
122. WELLS, L. J. *Anat. Record.* 94: 504, 530, 1946.
123. WELLS, L. J. *Proc. Soc. Exper. Biol. and Med.* 62: 287, 1946.
124. WENGRAF, F. *Z. Kinderheilk.* 30: 79, 1921.
125. WEST, J. R., H. W. SMITH AND H. CHASIS. Methods of Measuring Renal Clearance with Special Reference to Infancy and Childhood. Communication to the Fifth International Congress of Pediatrics. New York, 1947.
126. WILKINSON, E. Private communication. 1947.
127. WILKINSON, B. M. AND R. A. MCCANCE. *Quart. J. Exper. Physiol.* 30: 249, 1940.
128. WINDLE, F. W. The Physiology of the Fetus. Philadelphia and London: 1940.
129. WINKLER, A. W., T. S. DANOWSKI, J. R. ELKINTON AND J. P. PETERS. *J. Clin. Investigation.* 23: 807, 1944.
130. YLPPÖ, A. *Z. Kinderheilk.* 14: 268, 1916.
131. YLPPÖ, A. *Acta. Pediat.* 3: 235, 1923–24.
132. YOFFEY, J. M. Communication to the Anatomical Society. June 27, 1947.
133. YOUNG, W. F., J. L. HALLUM AND R. A. MCCANCE. *Arch. Dis. Child.* 16: 243, 1941.
134. YOUNG, W. F. AND R. A. MCCANCE. *Arch. Dis. Child.* 17: 65, 1942.

THE CHEMICAL, PHYSICAL AND MORPHOLOGICAL PROPERTIES OF ANIMAL VIRUSES

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THE DIVERSITY in the biological activities and effects of viruses is well known. The range of pathological lesions and host-cell reactions extends from rapid and total necrosis of affected tissues to the stimulation to uncontrolled proliferation of parasitized cells in virus tumors (96). This versatility of viruses as a group is not cause for wonder, for variety in effects is characteristic of the action of other types of infectious agents. Considering their size, however, there might appear to be less basis for conceiving a comparable diversity in the physical, chemical and morphological attributes of the viral agents. Indeed, from the beginning, there has been a tendency, still continued, toward simplification of concepts of these characters as well as of the biological nature of viruses. The increasing accumulation of data in recent years, however, is rapidly dispelling the illusions of simplicity implied in theories of the enzymic, genic or molecular character of the agents. As one virus after another has been examined, there is added the evidence of further differences and complexity.

Since 1930, several viruses affecting man, animals, plants and bacteria have been isolated and studied in purified preparations. The plant viruses, the findings with which have been discussed on many occasions (126, 7, 129), consist of protein and ribopentose nucleic acid in varying amounts, the latter constituting as much as 40 per cent of the tobacco ring spot virus (127). Only two shapes have been observed, those of the rod and the sphere, and some of the agents are of such uniformity in size and shape that crystals are readily formed. In contrast, the few human, animal and bacterial viruses studied are of greater complexity (121, 41, 9, 91, 10) in constitution than plant viruses and much more variable in external morphology. In addition there is evident an internal structure which likewise differs from one agent to another. It is with the findings obtained with the latter group of agents represented by the viruses of vaccinia, rabbit papillomatosis (119), equine encephalomyelitis (Eastern (140) and Western (77) strains), influenza (human types A (124) and B (33, 71, 34) and the swine type (118, 120)) and Newcastle disease (61, 27, 14) and with bacteriophages that the present paper is concerned.

CONSTITUTION

From the beginning the size range of viruses has been an obstacle not only to direct experimentation but to thoughts of the possible characters of these agents. The status of viruses in this respect has been, and still is in the minds of some, no different from that of organisms beyond or at the threshold of vision in earlier times (93).

It was hard to conceive of entities so small being able to contain the substances of life. Soon after tobacco mosaic virus was first filtered (51), Beijerinck (17) referred to the infectious agent as a contagious fluid. Many have regarded viruses as enzymes incapable of self-determination and formed only by the activity of the stimulated host cell. The experiments and arguments purporting to substantiate these views have been numerous even in recent years. Northrop (85) regarded a bacteriophage of a staphylococcus as an enzyme of sizes, determined by diffusion, varying from 10 to 100 mμ. Kalmanson and Bronfenbrenner (53) considered the T_2 bacteriophage of E. coli to consist of protein associated with only traces of phosphorus and to be enzyme-like in nature. Stanley (125), reporting for the first time the chemical purification of tobacco mosaic virus, called this agent a globulin of the nature of an autocatalytic enzyme.

With the results of more direct examination, some of these findings have not been confirmed and some of the views have languished. Wyckoff (145) could not demonstrate in the analytical ultracentrifuge the variation in the size of phage particles predicted by Northrop; Bawden *et al.* (8) found nucleic acid in the tobacco mosaic virus; and Hook *et al.* (49, 108) and Taylor (134) demonstrated the complexity in the constitution of the T_2 bacteriophage of E. coli, corroborating Schlesinger's (100–103) findings years before with a bacteriophage of E. coli.

The animal viruses exhibit constitutions lacking in but few, though important, respects the chemical constitution of organisms, consisting, in addition to protein and nucleic acid, of lipid components and one, the influenza virus, carbohydrate in excess to that found in nuclei acid. Differences in the proportions of the components among the various agents are profound. For example, the T_2 bacteriophage of E. coli contains 45 per cent of nucleic acid and the influenza virus only about 2 per cent; and differences in proportions are nearly equalled by differences in kinds of constituents. The component constitution of the animal viruses studied and that of the T_2 bacteriophage are summarized in table 1.

The rabbit papilloma virus, the smallest animal agent thus far examined, appears to be the simplest in constitution (11, 135). The nitrogen content, 15 per cent, is indicative of a large proportion of protein, estimated on fractionation as 90 per cent of the whole complex. Whether or not lipid is an integral constituent of the virus is subject to question; 1.5 per cent has been the largest amount extracted from preparations of the agent. The nucleic acid, difficult (135) to free from the whole complex, is relatively large in amount, about 8.7 per cent, and appears to consist wholly of the desoxypentose type.

Vaccinal elementary bodies, one of the largest and most intensively studied animal viruses, differ in some respects but possibly not fundamentally from the papilloma virus. The amount of protein, 89 per cent, is comparable to that in the papilloma virus; the remainder of the complex is almost equally divided between nucleic acid and lipid. Many workers have contributed to the knowledge of the constitution of vaccinal elementary bodies. Protein, lipid and carbodhydrate were demonstrated by Hughes, Parker and Rivers (50); phosphorus was determined and nucleic acid of the desoxypentose type was recognized by McFarlane and Macfarlane (74) who also obtained tests for glucosamine. McFarlane *et al.* (75) found 8 per cent carbohydrate,

indicating the presence of more carbohydrate than that bound in nucleic acid. Hoagland and his associates (42–48) in studies of carefully purified virus confirmed these findings qualitatively. In contrast, however, the value representing the carbohydrate content of thoroughly washed bodies (43) was reduced to 2.8 per cent; and the amount of nucleic acid (42) was 5.6 per cent, apparently all of the desoxypentose type.

The lipid fraction, 5.7 per cent, was fractionated (43) into phospholipid, neutral fat and cholesterol. While the former two were considered to be actual constituents of the virus, questions were raised regarding cholesterol. This material could be

TABLE 1

	WHOLE COMPLEX				LIPID				NONLIPID					REFERENCES
	C	N	P	Carbohydrate	Total	Phospholipid	Cholesterol	Neutral fat	Total	Protein	Carbohydrate	Nucleic Acid		
												INA	RNA	
Broth bacteriophage	42.0	13.5	4.84	13.6	2.6	0	0	2.6	97.4	50.6	13.1	40.3	6.6	134
Synthetic medium bacteriophage	42.3	13.3	5.22	11.7	1.8	0	0	1.8	98.2	52.4	11.2	44.6	1.3	134
Vaccinia	33.7	15.3	0.57	2.8	5.7	2.2	1.4	2.2	94.0	89.0	2.8	5.6		42, 43
Papilloma	49.6	15.0	0.94	6.5	1.5				98.5	90.0		8.7		11, 135
Equine encephalomyelitis (Eastern St.)	62.2	7.7	2.2	4.0	54.1	35.0	13.8	9.6	53.0	49.1	7.2		4.4	237
Influenza A (PR8 Strain)	53.2	10.0	0.97	12.5	23.4	11.3	7.0	5.1	77.5	65.0	7.3	1.5	?	133
Influenza B (Lee Strain)	52.7	9.7	0.94	13.1	22.4	11.2	3.7	7.2	76.4	63.6	9.4	1.2	?	133
Swine Influenza	51.4	9.0	0.87	10.0	24.0	10.7	5.7	7.7	77.6	67.6	10.0	+	?	133
Broth medium bacterium	49.1	13.2	2.72	12.5	7.75	7.75	0	0	92.3	67.9	12.5	5.2	19.1	134
Synthetic medium bacterium	49.0	13.2	2.66	11.6	9.11	9.11	0	0	90.9	67.7	11.6	2.4	20.9	134

All values are percentage dry weight of the whole complex.

extracted with ether from lyophilized bodies further dried over phosphorus pentoxide without loss of infectivity. Inasmuch as approximately 98.5 per cent of the bodies were already inactive (10) due to the drying treatment, the findings of the authors are inadmissable as evidence that cholesterol is not an integral constituent of vaccinia virus. The phospholipid was of the lecithin type containing phosphorus and nitrogen in a 1:1 ratio.

Further examination of the vaccinal elementary bodies showed the presence of 0.05 per cent copper (46) which acted as the catalyst in the oxidation of cysteine. Biotin (45) and flavin-adenin-dinucleotide (47) were present. In the search for enzyme constituents, McFarlane and Salaman (70) demonstrated phosphatase and

catalase, and in addition to these, Hoagland *et al.* (43) found lipase. The latter authors also found that the 3 enzymes could be adsorbed in large amounts on the bodies, and, consequently, the place of these enzymes in the constitution of the virus was equivocal.

The two strains, Eastern and Western, of equine encephelomyelitis virus exhibit unusual and extreme properties (136, 132). Protein and lipid are approximately equally divided, and the nucleic acid, only 4.4 per cent, is entirely of the ribopentose type in contrast to the desoxypentose type of the vaccinia and papilloma agents. The lipid component consists chiefly of phospholipid, 35 per cent, of the diamino-monophosphatide type. Both cholesterol and neutral fat were found. There were no constitutional characters differentiating the two strains.

Among the animal viruses thus far studied, the agent of equine encephelomyelitis is unique in its content of only ribopentose nucleic acid. Both desoxypentose and ribopentose types have been reported to contribute to the structure of the influenza viruses and to that of the T_2 bacteriophage of E. coli. All of the plant viruses examined thus far contain only ribopentose nucleic acid.

The influenza viruses A and B have been studied by several investigators (137, 116, 133, 130, 131, 55, 56, 58) with closely similar results. In constitution the swine influenza virus (139) does not differ greatly from the two types affecting man. The three types of influenza viruses contain about 25 per cent lipid, relatively very small amounts of nucleic acid and carbohydrate in excess of that found in the nucleic acid.

Difficulties have been experienced in the analysis of the small amount of the nucleic acid component. In the earlier studies (137, 116, 139, 133), only the desoxypentose type was reported; later, Knight (55, 56, 58), taking exception to Taylor's (133) methods, reported positive tests for the other type, ribopentose, of nucleic acid. While the desoxypentose type is undeniably there, further confirmation is needed in the case of the ribopentose type.

Though discrepancies were apparent in the amounts of carbohydrate reported by Taylor (133) and Knight (58), the findings uniformly revealed the presence of far more than could have been bound in the minute quantities of nucleic acid. With the carbazole method, Taylor (133) demonstrated mannose or glucose-galactose or both in complex form. These findings were corroborated by Knight (58) who also identified glucosamine. Knight (59) has recently studied the amino acid composition of influenza viruses A and B, reporting differences between the two agents believed to be significant. On the basis of the results of precipitin studies, Knight has suggested (57) that influenza virus may contain components antigenically identical with normal components from chorio-allantoic fluid or mouse lung, depending on the host.

Schlesinger (101–103) was the first investigator to concentrate and purify a virus for chemical analysis. The agent was a bacteriophage of E. coli which he found to contain protein and fat. Phosphorus was also found in a large amount in the initial studies but related only later to the presence of nucleic acid. Northrop (85) found a high content of phosphorus in his bacteriophage of the staphylococcus, but Kalmanson and Bronfenbrenner (53) could demonstrate only traces of the element in the T_2 bacteriophage of E. coli concentrated by ultrafiltration.

The T_2 bacteriophage (25) of E. coli, like the animal viruses, consists (134) of

protein, nucleic acid and lipid, and the amount of carbohydrate present is compatible with the quantity of nucleic acid. One major difference from the animal viruses is the amount of nucleic acid, about 45 per cent. Both desoxypentose and ribopentose types have been reported, the former greatly in preponderance, but Cohen and Anderson (23) have denied the presence of the ribopentose type. To some extent, possibly, the differences may have been related to differences in techniques. By the method of Schmidt and Thannhauser (104), Taylor (134) found 40.3 per cent desoxypentose nucleic acid and 6.6 per cent of the ribopentose type in the phage cultivated on the host grown in broth medium. Only 1.5 per cent of the latter and 44.6 of the former were found in the phage obtained from lysates of the host grown in synthetic medium. The tryptophane method of Cohen (22) did not give a positive test for ribopentose.

A fundamental difference between this bacteriophage and the animal viruses is in the qualitative content of lipid. Whereas phospholipid, cholesterol and neutral fat are found associated with all of the animal viruses except the agent of rabbit papillomatosis, which has not been examined in this respect, the bacteriophage contains only neutral fat, neither cholesterol nor phospholipid being present. The total amount of lipid is small, only about 2 per cent.

Experiments with bacteriophage afford singular opportunities for the study of the relation of virus constitution to that of the host. In microchemical analyses similar to those made on the bacteriophage, fractionations (134) were carried out on the host cell, E. coli, cultured in broth medium and synthetic medium. Though in certain respects the structure of the parasite resembles that of the host, in others there are striking differences. While both consist of protein, nucleic acid and lipid, the lipid differed vastly from that of the virus, consisting wholly of phospholipid of which the virus contained none. Neither cholesterol nor neutral fat, the latter comprising the lipid of the virus, could be demonstrated. Both types of nucleic acid were found but the proportions were the reverse of those in the virus, ribopentose nucleic acid being present in the larger amounts in the bacterium. These findings constitute evidence of physiological independence on the part of the bacteriophage in the processes of reproduction; for the bacterium is able to synthesize phospholipid, none of which is found in the phage and is either unable to or does not form neutral fat which is a constituent of the bacteriophage.

Concentrates of the virus of Newcastle disease reveal (24) a constitution not greatly different from that of the influenza viruses. While the results obtained thus far have been with obviously impure material, no unusual characters of virus constitution were noted.

MORPHOLOGY

Some of the viruses are of a size within the limits of visualization with the light microscope. For many years before viruses were separated into a special category, specific structures, the inclusion bodies, had been seen in the cells of certain diseased tissues. Later, much smaller coccoid bodies were observed in some instances. Borrel (18) in 1904 discovered in fowl pox lesions the barely visible bodies which Woodruff and Goodpasture (142, 143) subsequently found were contained within the inclusion or Bollinger bodies. These workers provided evidence of the identity of the Borrel

bodies with the virus of fowl pox. Similar entities found in tissues affected with other diseases—vaccinia, molluscum contagiosum and psittacosis—have been considered to be the respective viruses and regarded as microorganisms.

Though much was learned by these methods, the various elementary bodies were just visible in the light microscope, and the data were extended but little by use of the ultraviolet light microscope. The enormously greater magnifications of the electron microscope, affording a useful range up to nearly 100,000 diameters, including photographic enlargement, have revealed shape, size and internal virus structure. Dependent on differential absorption of electrons by the various portions of the substance of the virus, electron micrography leaves much to be desired with respect to contrast at the periphery of the particle and internal regions of segregated material. Thus far no way has been found to increase differentiation by staining, though calcium chloride (111, 137) is useful in increasing contrast at the surface. Of extreme value has been the shadow technique (83) applied to the study of viruses (141, 148). The three dimensional effect thus given affords a good idea of the shape of the virus.

Some viruses are essentially spherical. This is particularly true of certain plant viruses, those of bushy stunt of tomato (92) and bean mosaic disease (92, 147). These agents appear extraordinarily uniform in shape and size, properties associated with their capacity to form crystals.

Among the animal viruses, the agents of rabbit papillomatosis (109, 113) and equine encephalomyelitis (111) appear to be spherical or nearly so. Images of these two agents are circular in conventional electron micrographs and seem highly uniform in shape and size. However, the boundaries of the images are indistinct and small variations in size could not be determined if they existed. In shadowed preparations of the papilloma virus, the shadows cast are not as long as those expected of spheres. The shadows cast by the bushy stunt virus (92) indicate a more nearly spherical shape, a possible indication that either the plant virus is more nearly spherical under natural conditions or the changes occurring during drying differ with the two agents. Shadowed preparations of the equine encephelomyelitis virus have not been obtained.

The influenza viruses, A (137, 26), B (116) and the swine agent (139), differ greatly from the papilloma and equine encephalomyelitis viruses. The three types of influenza virus, indistinguishable in electron micrographs, are neither spherical nor uniform in size. In conventional micrographs the images vary in shape from circular to oval and elliptical. Many seem bean-shaped. The evidence of considerable variation in size is corroborated by the diffuse sedimentation boundaries in the analytical ultracentrifuge. Shadowed preparations show forms varying as the images in conventional micrographs. Some flattening of the virus was evident. Mosley and Wyckoff (82) reported filamentous forms, but there is no evidence that they are significantly related to the virus.

A still greater departure from the spherical shape is seen with vaccinia virus. Conventional micrographs (39, 113) show rectangular images of fairly uniform widths but of definite variation in length. Green, Anderson, and Smadel (39) construed their pictures as indicating a brick shape. There is reason, however, to consider the particles as short rods with rounded ends. In some micrographs there occur round images of a diameter equal to that of the width of the rectangular images. Such

images are of high contrast and may indicate particles standing on end. Micrographs of shadowed preparations (113) show again rectangular or biscuit-shaped images. The relatively short shadows seen indicate a marked flattening of the bodies, due, certainly in large part, to much shrinkage on drying. The superior surface of the shadowed particles is not smooth. Instead, there are rounded, mound-like elevations which look like protrusions of internal structure elevating the surface. Substantiating the evidence of rod-forms seen in conventional micrographs, are round images with relatively very long shadows, indications of bodies standing on end. In some conventional micrographs, there are seen outline or 'ghost' forms (39) which can be produced by treatment with alkali. These forms look like the empty shells of the bodies from which the internal contents have escaped.

The elementary bodies of fowl pox (40, 19), canary pox (99, 40), molluscum contagiosum (99, 19), ectromelia (99, 19) and myxoma (19) appear in electron micrographs to be forms resembling those of vaccinia virus. Shadowed preparations of fowl pox virus showed the unevenness of surface contour like that seen with vaccinal elementary bodies (113).

Some of the bacteriophages are tadpole-shaped (98, 68, 69, 113, 49). To one end there is attached broadly a tail structure which, in the T_2 bacteriophage of E. coli (49), terminates in a brush- or disc-like expansion. In most cases the tail has the appearance of rigidity, lying in a line straight with the long axis of the head. In preparations which have been sedimented repeatedly, some of the tails lie at a sharp angle with the head, and many tails are separated from the heads. A most interesting phenomenon is the occurrence of ghost forms with the outline of the whole particles but without evidence of the high electron-absorbing material of the head. Such forms can be produced (4) in large numbers by sonic vibration and are probably analogous to the ghosts seen in preparations of vaccinia virus treated with alkali. These findings provide evidence of a limiting structure containing the internal substance of the phage.

In shadowed preparations, the headpiece appears to be a short rod with conical ends and there is evidence of flattening possibly occurring during preparation of the mount. An interesting appearance is seen (49) in some shadow micrographs of particles washed with distilled water and lightly coated with metal. The images under these conditions reveal a roughened, hourglass surface contour caused, evidently, by protrusion into the surface of the double internal structure described above. Other bacteriophages appear to be essentially spherical, as judged (98, 68) from the circular images in conventional electron micrographs. Such forms do not show the presence of tails.

At the extreme of forms removed from the sphere is the shape of the virus of Newcastle disease (24, 5). This agent is a relatively very long—about 700 mμ—slender, sperm-shaped entity. The head, about one fifth the length of the whole is narrow, oblong or nearly ellipitcal, and one end is continuous in a slender, frequently curved tail which tapers sharply at the distal end. The various curves of the tail-piece give the impression that it might be motile. In some micrographs, confusing forms are evident. There are ring shapes, shapes open in the center and great balloon forms of low contrast. These bizarre shapes are seen especially under conditions

which might result in damage to the particles, such as that caused by repeated centrifugation or suspension in distilled water. The tails are frequently torn from the heads, leaving a frayed region or only a very short remnant of the tail at one end of the head. The ring and doughnut forms have been construed as tails curved upon themselves and the balloon shapes as heads which have taken up water and become swollen. Such swollen forms of low contrast have been seen frequently in influenza virus preparations in distilled water. Pleomorphism of the Newcastle disease virus has been suggested (5), but too little is known to subscribe to this concept.

INTERNAL STRUCTURES

Some of the viruses are very difficult to picture in electron micrographs because of the low electron-absorbing power of their constituents. For this reason, though internal structure is visible in all of the agents causing disease in animals, clarity has not been obtained. The use of calcium chloride in dilute solutions is valuable in enhancing the contrast of the external particle outline in some instances, but internal structure is proportionately obscured. All save external contours is obscured in shadowed preparations.

In images of the papilloma virus (109), there is present a single, circular region of contrast higher than that of the remainder of the particle. The boundary of the region is not well limited but fades into the surrounding substance of the particle. The papilloma virus image has the appearance of a cell with nucleus and cytoplasm somewhat resembling a small lymphocyte. Similar internal structures are present (111) in the images of freshly prepared equine encephalomyelitis virus. In images of particles that have stood in Ringer solution for several days, the internal structure shows up better; here, the substance may be well outlined, circular or oblong, and two regions of segregation may be seen. Thus, under these circumstances, internal structure is not only present but may be varied in shape, size and number of regions in a single particle. Vaccinia virus reveals (39, 113) the presence of one to five regions of high contrast. These are round in conventional micrographs, are scattered through the particle and constitute a relatively small part of the substance of the particle. The mound-like protrusions in the surface seen in shadowed preparations may well represent these structures which may shrink less on drying than the remaining substance of the particle.

The internal structure of the influenza viruses (137, 116, 139, 26) appears to be a single structure of indefinite outlines, more often than not, eccentrically placed. In the bean-shaped particle, the internal material lies near the hilum.

Some of the bacteriophages (98, 68, 69, 113, 49), notably T_2 of E. coli, show unusually clearly the presence of structure within the headpiece. This material, absorbing electrons with high degree, is bow-tie or dumbbell shaped and occupies a large part of the head, there being but little surrounding substance. It was noted above (49) that the surface of phage particles coated with metal under certain conditions is roughened, giving evidence of the internal structures protruding, mound-like, into the surface contour.

The particles of Newcastle disease virus contain (24) in the headpiece internal structure of variable characters. In some the material seems single, in others double

with considerable space between masses. These masses, or portions of them, appear to reach in some instances into the proximal region of the tailpiece.

Though the findings of virus morphology are fragmentary, it is obvious that the particles have the appearance of organisms. It is supposed that the high electron absorbing material contains the nucleic acid present, for nucleic acid carries the atoms of the highest atomic numbers such as phosphorus. The internal materials look like cell nuclei surrounded by protoplasm. In vaccinia virus, which contains a small amount of nucleic acid, the internal structure is small in comparison with the rest of the particle. The opposite is true for the T_2 bacteriophage, for the particle is nearly full of the inner material.

ULTRACENTRIFUGAL CHARACTERS

Those properties of particles determining their rate and manner of sedimentation are size, shape and density. Knowledge of the size or density, provided the shape is not too complex, permits calculation of the other from the sedimentation constant. The sedimentation constant, corrected for the viscosity, temperature and other factors concerned with the suspending medium, is characteristic of the particles examined. Sedimentation diagrams provide a basis for judging the uniformity of particle characters and certain aspects of the purity of the preparations. In the period of a few years before the introduction of the electron microscope, most of the judgment of the physical characters of viruses in this respect was, and frequently still is, given a significance far beyond the limits of justification. Conservatively interpreted, however, sedimentation diagrams provide relatively accurate estimates of many properties of viruses.

If all of the particles of a given preparation are of exactly the same size, shape and density, all will sediment at the same rate, and the descending boundary will be very sharp and clear. Variation in these characters will lead to proportionate diffuseness of the boundary. Two or more different groups of particles in the same preparation, if close enough in sedimentation properties, will give rise to two or more boundaries. It should be noted at the outset that the characters of the sedimentation diagram have no significance whatever with respect to the ultimate nature of the sedimenting particles.

Sharp sedimenting boundaries are seen with several of the animal viruses. The bacteriophage T_2 of E. coli gives an exceedingly sharp boundary (49, 108), indicating a high degree of uniformity in the size, shape and density of the particles. A bacteriophage of staphylococcus concentrated by Northrop (85) and examined by Wyckoff (145) likewise showed very sharp boundaries. With some preparations, slightly less sharp boundaries are seen with the papilloma (13, 11, 84) and both Eastern and Western strains of equine encephalomyelitis viruses (111). In both instances, though relatively high degrees of particle uniformity are evident, there is slight diffuseness.

Vaccinia (12, 3, 89) and influenza viruses (35, 36, 114, 65) give diffuse boundaries indicating a lack of uniformity of sedimentation characters. It is likely that the diffuseness observed is due mainly to differences in particle size, since variations in this character of both viruses are seen in electron micrographs. In the instance of the influenza viruses, the spread of sedimenting boundaries obtained (114) with the

Lamm scale method is closely similar to the spread in size measured in electron micrographs. The whole significance of the very diffuse boundary given by the Newcastle disease virus (24) cannot be judged since it was known that preparations of the agent were not pure. In all instances there appears to be a close correlation between sharpness or diffuseness of sedimenting boundaries and the uniformity, or lack of it, in the sizes of virus particles observed in electron micrographs.

The sedimentation constants of the animal viruses are given in table 2. There is seen a variation from S = 265 × 10^{-13} for the equine encephalomyelitis virus (111) to S = 4910 × 10^{-13} for the vaccinia virus (89). The sedimentation constant for a given preparation of a virus is reproducible (66) with high accuracy even in different laboratories. Nevertheless, different preparations of an individual agent may yield values of rather wide variation. For the papilloma virus variations (84) have been seen between the limits of about S = 265 to S = 290 × 10^{-13}. It seems evident that

TABLE 2

	SEDIMENTATION CONSTANT	DENSITY IN AQUEOUS SUSPENSION	SIZE SEDIMENTATION VELOCITY DATA	SIZE ELECTRON MICROGRAPHS	PARTIAL SPECIFIC VOLUME	WATER CONTENT	REFERENCES
	× 10^{13}		mμ	mμ		% by volume	
Vaccinia	4910	1.16	236–252	222 x 284	0.793		89, 122, 106, 70
Papilloma	278	1.133	66	44	0.669	58	112
Equine encephalomyelitis (Eastern strain)	265				0.839		136
Influenza A (PR8 strain)	742	1.104	116	101	0.822	52.0	115
Influenza B (Lee strain)	840	1.104	124	123	0.863	34.5	115
Swine Influenza	727	1.100	117	96.5	0.850	43.3	115
T_2 bacteriophage (broth)	1022 695			80 x 100	0.655		108, 134

differences of this magnitude are not due to experimental error and that, while the particles of one preparation may be highly uniform, variation must occur either in the sizes or the densities of particles from different sources. Like differences have been observed (12, 3, 89) with other viruses such as vaccinal elementary bodies of different strains and preparations.

A most interesting behavior of the T_2 bacteriophage of E. coli is the occurrence (108) of two separate boundaries depending on the conditions of the experiment. This virus suspended in saline media of pH above 5.8 gives a very sharp boundary of S = about 700 × 10^{-13}. In media of pH below 5.8, there is seen a similarly sharp boundary of S = about 1000 × 10^{-13}. With proper balance of pH near 5.8, both boundaries may be seen at the same time. Both boundaries are associated with full phage activity, and the transition from the one state to the other occurs without change in infectious capacity. The boundary of S = about 1000 × 10^{-13}, occurring in the acid range, can be produced in the alkaline region by the presence of calcium. These phenomena have been interpreted (108) as related to the state of aggregation or dispersion of particles of the tadpole shape.

DENSITY

The density of virus particles as they exist in aqueous media under natural conditions has been a most difficult property to investigate. Theoretically, one of the simplest methods would be the study of change in sedimentation rate with change in density of the suspending medium. A number of such studies has been made from time to time with both bacteria (97) and viruses—bacteriophage (100, 30, 76), vaccinia (15, 16, 31, 122, 75), influenza virus A (31, 67) and the swine influenza virus (115)—but the results have been difficult or impossible to interpret. In all of the earlier studies, materials such as sodium chloride and sucrose were employed for attaining the desired changes in the density of the suspending media. Under these conditions, the density of the virus increased (122, 67, 115) with increase in the concentration of the solute. Such a behavior was considered to be due, possibly, to osmotic effects and loss of water from the particles. A material offering possibilities for circumventing such an effect is bovine serum albumin, which has a molecular weight presumably in the region of 70,000 and, consequently, a negligible osmotic pressure in the range of concentrations desirable for the experiments.

Studies with the three types of the influenza viruses and on the papilloma virus have revealed linear relationships between the sedimentation rate and the density of the suspending medium. There was thus no evidence of change in density of these viruses due to the effect of bovine serum albumin. The values obtained in this way, table 2, were 1.104, 1.104, and 1.100 for the influenza viruses A and B and the swine virus (115), respectively, and 1.133 for the papilloma virus (112). These values are much lower than those for vaccinia, 1.16 (122), and influenza virus A, 1.148, obtained with sucrose. The value 1.148 was derived (115) mathematically from the curve of Lauffer and Stanley (67) from which they inferred the value 1.1. It is notable that the density range involved in the second and third decimal places, that is, from 1.100 to 1.199, represents a range of approximately 100 per cent in the sedimenting properties of the virus based on density. From these experiments it is evident that values obtained by materials such as sucrose are of use only in the estimation of possible upper limits. Bovine serum albumin is, unfortunately, unsuitable for density work with some viruses; neither the bacteriophage T_2 (106) nor the tobacco mosaic virus (64) remain in suspension in solutions of the material in the density range required.

Vaccinia virus suspended in concentrated sucrose solutions increases in density for a time and then, on continued exposure to the solution, decreases in density (122). A similar behavior has been observed for the influenza virus A (115). This property suggests that these agents are limited by a semipermeable structure or membrane. In the instance of vaccinia virus there is evidence of such a structure in electron micrographs, and it will be recalled that the headpiece of the T_2 bacteriophage is enveloped in a structure from which the contents of the head may spill to leave ghost forms.

The partial specific volumes determined in the pycnometric studies of the animal viruses thus far investigated, given in table 2, vary from 0.669 for the papilloma virus to 0.863 for influenza virus B. With these values and a knowledge of the density of the particles in aqueous suspension, the water contents, table 2, for the papilloma (112) and influenza viruses A and B and the swine influenza virus (115) are 58 and 52.0, 34.5 and 43.3 per cent, respectively, by volume. Lauffer and Stanley (67) assumed a water content of 60 per cent for influenza virus A.

SIZES

The general size range of viruses is now well known. The ultrafiltration studies by means of graded collodion membranes (29) have given close approximations with viruses which are spherical or nearly so. Serious errors are encountered, as for example with the Newcastle disease virus, where the particles are long. In this case, though, the values 80 to 120 mμ (21) were close to the widths of the heads of the sperm-shaped particles observed (24) in electron micrographs of this virus. A discussion of sizes and methods of study was given by Markham, Smith and Lea in 1942 (72). The more recent values obtained from ultracentrifugal and electron micrographic measurements are in farily close agreement from one laboratory to another. While such values are undoubtedly not precise, they represent approximations close enough for practical uses. In most of the instances, the values can represent only an average for the particles of those viruses which vary in size.

The sizes of the viruses under discussion here are given in table 2. Average values obtained from electron micrographs have been reported for the viruses of molluscum contagiosum, 255 × 178 mμ (99) and 302 × 266 mμ (19); fowl pox, 332 × 264 mμ (99); myxoma of rabbits, 287 × 233 mμ (99); canary pox, 311 × 263 mμ (99); and ectromelia, 232 × 172 mμ (99) and 300 × 210 mμ (19).

ELECTROPHORESIS

Another criterion of virus character and behavior is the manner of migration of the particles in an electrical field. Electrophoresis experiments (1) in the earlier work with various types of instruments gave little quantitative information, in part, because extraneous material in the preparations far outweighed the amount of virus. Significant experiments with some viruses became possible with development of the Tiseleus apparatus and access to purified preparations. Not all viruses are adaptable to the conditions of study with the instrument because of instability, turbidity of the preparation or other reasons. There has been some interest in possibilities of virus purification by the method but little has been done in this respect.

Several investigators have worked with vaccinal elementary bodies. McFarlane (73) observed boundaries which were not sharp and which exhibited a streaming effect that was thought to be due to endosmosis. Other workers (117, 123), however, demonstrated that the phenomenon was due to density of gradient effect that decreased when the virus concentration was raised to 0.5 per cent and disappeared when serum protein was present. Under these conditions sharp boundaries were obtained. With the Northrop-Kunitz (86) microcataphoresis method, the isoelectric point appeared (12) to be in the region of pH 4.36 to 4.6. At pH 7.9 the mobility in the Tiselius electrophoresis apparatus was about 10×10^{-5} cm/sec. per volt/cm. The virus is inactivated relatively rapidly below pH 5.5, which is still well above the isoelectric point.

The clearest and sharpest boundaries for an animal agent were those obtained (110, 107) with the virus of rabbit papillomatosis. The virus is stable both above and below the isoelectric point, which was observed (13) to be about pH 5.0 in microcataphoresis experiments. In the pH regions adjacent to 5.0 the virus agglutinates, but studies could be made on active virus at pH 3.77 to 4.0 and 6.54 to 7.77. In the

acid region there was rapid cathodic movement of an exceedingly sharp single boundary. Seemingly more diffuse boundaries moved much more slowly toward the anode in the alkaline region. Analysis of the Svensson curves in this region, however, showed that the heterogeneity values (107), H, were similar in the two regions. The findings were considered to indicate a high electrophoretic homogeneity of the virus in the acid region and but little less homogeneity in the alkaline range. The mobility was from 3.85×10^{-5} cm/sec. per volt/cm. at pH 3.78 to -1.03×10^{-5} cm/sec. per volt/cm. at pH 7.77, and the points were compatible with a smooth curve including the isoelectric point at pH 5.0.

Sharp single boundaries are obtained with influenza virus A after special efforts at purification (79). Otherwise, a second boundary related to contaminating extraneous particles presumably derived from chorio-allantoic fluid is seen. The isoelectric point, in microcataphoresis experiments, was at pH 5.0. In the alkaline region, where the virus was stable with respect to infectivity, the mobilities were from -0.67 to -0.66×10^{-4} cm/sec. per volt/cm. between pH 9.3 to 7.4. At pH 3.0 and 2.8, the virus, which must have been inactive, gave mobilities of 0.87 and 0.97×10^{-4} cm/sec. per volt/cm. All of the points, nevertheless, fell on a smooth curve drawn through the isoelectric point. Influenza virus obtained (56) from mouse lung exhibited a more variable behavior.

STABILITY

A relatively uninvestigated field is that related to the stability of animal viruses under various physical and chemical conditions. This property has been most studied in relation to the effects of hydrogen ion concentration, principally in order to know how to protect and preserve the agents during and after the purification procedures. The results of such studies have been reported for vaccinal elementary bodies (12), the papilloma (13), equine encephelomyelitis (32, 138) and influenza viruses (78, 79) and the T_2 bacteriophage (108) of E. coli. In general the animal viruses are unstable below about pH 5.5 to 6.0 and above pH 9.5. The papilloma virus is a notable exception for, like some of the plant viruses, it is stable below the isoelectric point. Indeed, the greatest stability is seen in the acid region from about pH 3.5 to neutrality. It is a general finding that particle integrity enjoys a wider range of stability than the quality of infectivity.

A field but little studied yet is concerned with the effects of salts, both of kind and concentration, on animal virus stability. The papilloma virus agglutinates at salt concentrations much below 0.05 M but disperses readily and is stable in 2 per cent NaCl solution. Vaccinal elementary bodies agglutinate (87, 12) and are damaged (12) in 0.9 per cent NaCl solution and in distilled water. Buffers—phosphate, citrate (87) and others (12)—of 0.005 M concentration appear optimum. Equine encephalomyelitis virus is more stable (136) in Ringer solution than in NaCl solution (144) and, perhaps, more so in the presence of cysteine (6). The virus agglutinates in distilled water. The influenza viruses are stable in Ringer solution (137, 139, 116) or 0.1 M phosphate buffer (54). Damage occurs in phosphate buffer of 1.0 M or 0.001 M concentration or in distilled water, in which the virus is not dispersed (133). A rather detailed study (60) showed that this virus was not

damaged by several reducing agents, except ascorbic acid; was inactivated quickly by phenol, 0.5 N; and was weakly affected by sulfathiazole sodium.

COMMENT

There have been many discussions (2, 38, 52, 37, 93, 94, 7, 128, 80, 146) in recent years concerned with the nature of viruses, most of which were written before much progress had been made in the study of the animal agents. None of the present day views is new in principle though some variation has occurred in the details. These concepts, familiar to all who have followed the literature describing the physicochemical studies on plant viruses, have been reviewed in detail by Rivers (94) and were discussed critically by Bawden (7). In brief, there are those who believe that viruses are molecular entities and others who consider them as organisms. Recently Burnet (20) has discussed the biological reasons for regarding viruses as organisms. Stanley has been the outstanding proponent of the molecular theory. Precisely what the term molecule is intended to mean, aside from the strictly chemical implications, is difficult (94) to judge from Stanley's discussions. From time to time there have been reiterated (128) the views of many writers extending through the gamut of autocatalytic enzymes and molecular nucleoproteins to comparisons of viruses with genes. Regardless of what has been meant, many have inferred that the molecular view has been synonymous with the concept that viruses may be inanimate 'chemical agents' (95) though some authors have spoken directly of 'living molecules' (62).

Belief that some of the plant viruses are molecular is based on interpretations (62, 63) with respect to homogeneity judged on a number of criteria, uniformity of chemical findings, sharp sedimentation boundaries, sharp electrophoretic boundaries and the capacity to form crystals. It is very clear that, although high degrees of uniformity are evident within the limits of the methods employed, the criteria are not of sufficient precision to permit the conclusions drawn. There is no doubt that many of the smallest viruses must be of relative quantitative simplicity in structure because of the limitations of size, but that any virus consists of a single compound "made up of the same number of atoms arranged in the same way" (62) not only has not been established but is incompatible with the known variability and adaptability of viruses with changing conditions (20). Of course, homogeneity is not an essential criterion of molecular structure, but it is the basis which has been employed for judging the plant viruses to be molecules. It is obvious, however, that on the basis of homogeneity, the animal viruses are not molecular. If a molecule is just any unit specifically organized to perform a given function or which constitutes a stable aggregate (88), then viruses, as well as bacteria and all cells, might be so designated.

Actually, whether some of the smallest viruses may approach molecular proportions is not of fundamental importance with respect to the principal questions of the biological nature of these agents—whether they are to be classed as a special category of living organisms, their modes or reproduction and the relations of their physiological activities to those of the host cell. If the term molecule is not meant to imply an inanimate nature of viruses, the concept immediately loses significance and meaning with respect to the place of viruses in the biological scale.

It would seem that the findings with the animal viruses and the bacteriophage

should give little reason for confusion. The data obtained present a series or system continuous with the findings of other known entities, organisms and cells. The size range of animal viruses studied in some detail extends from about 250 mμ—vaccinal elementary bodies—to about 60 mμ—the papilloma and equine encephelomyelitis viruses. Between these limits of size there is no evidence of a break or gap in fundamental constitution, and in no instance, except for the size of the agents and the physical behavior of particles of this size, have the data diverged from the pattern of the qualitative character of organisms and cells. A specific comparison is that between the T_2 bacteriophage and its own host, E. coli. In this particular case qualitative component differences between the virus and the host cell indicate autonomy of the former in the process of reproduction. The nucleic acid content of the animal viruses varies from 8.7 per cent in the papilloma virus to about 2 per cent in the influenza viruses. In certain streptococci (105) the amount of nucleic acid is in the range of about 20 per cent, a value similar to that for E. coli. The content, about 45 per cent, of nucleic acid in the T_2 bacteriophage is considerably higher than that of its host and of these streptococci but is comparable with the amount in sperm and thymus gland cells (81, 80). It is possible that bacteriophages in general may be characterized by high contents of nucleic acid since the agents of this group from several sources contain large amounts of phosphorus. It is apparent that the physico-chemical findings with these viruses have closed no gaps between the animate and the inanimate; instead a known field has been broadened, and the problem of the biological nature of infectious agents of this size remains as great as ever.

The results of the analyses present no evidence confirming the belief that viruses may represent or be similar to 'genes' (28, 2). On the contrary the proportions of nucleic acid, and consequently 'genic' material, in the animal viruses are in general smaller and in no instance greater than those of organisms or cells (80). The internal structure, presumably representing condensations of nucleoprotein and a nuclear apparatus, is small in comparison with the whole particle; and there are clear regions corresponding to the cytoplasmic structures of organisms and cells. In the bacteriophage, which contains much nucleic acid, the internal material occupies a large part of the particle. Evidence of some sort of limiting peripheral structure having the appearance of a wall and the behavior of a semipermeable membrane in some instances is reason to doubt 'nakedness' of the particles. Rather than such a concept, it appears likely that viruses contain a proportionate amount of determinative material set aside within the virus cell in a unit not differing fundamentally from the analogous units of other organisms.

The group of animal and bacterial viruses thus far studied, though small in number, exhibits a diversity in chemical constitution which may well approach the limits to be expected for all of the agents of these two groups. There is the possibility that some of the agents of animal and bacterial disease, like the plant viruses, will be devoid of lipid. In neither the T_2 bacteriophage nor the papilloma virus is fat a large component, but if the fat is regarded as a vital part of these two viruses, all of the agents studied in both groups are complexes consisting of protein in association with nucleic acid and lipid and, in one instance, with carbohydrate in excess of the amount bound in nucleic acid. Reference to these agents as nucleoproteins, as frequently

practiced, is confusing and misleading, for the lipid is unlikely to be a constituent of nucleoprotein, and, further, there is no evidence that all of the protein and nucleic acid are bound in a single compound. With the information now at hand, such a usage of the term nucleoprotein is no more warranted with respect to viruses than it would be to refer to a bacterium, a liver or an oyster as a nucleoprotein.

Illusive reasonings are no longer provocative or helpful in the field for nearly everything has been said. The tools for further study are at hand, and numerous directions for new investigations are obvious. Many viruses must yet be purified, if no method is devised for their culture in simple media, before enough will be known to permit classification on a physico-chemical basis. Work in the field of the immunochemistry of viruses (57), which may tell much of virus structure and relationships, has barely begun. The usefulness of the electron microscope in physiological and morphological studies of viruses as well as bacteria is only beginning to be explored (148). Only a few investigations have been made of the physiological relations of virus and host-cell (25). Beginnings have already been made in the developments of techniques of the electron micrographic study of some aspects of virus growth and reproduction (149). As Burnet (20) has suggested, there is but one way to settle the questions, and that, undoubtedly will be the hard way of gathering the experimental data.

REFERENCES

1. Abramson, H. A., L. S. Moyer and M. H. Gorin. *Electrophoresis of Proteins and the Chemistry of Cell Surfaces.* New York: Reinhold Publishing Corp., 1942.
2. Alexander, J. and C. B. Bridges. *Science* 70: 508, 1929.
3. Amies, C. R. *J. Path. and Bact.* 47: 205, 1938.
4. Anderson, T. F. *J. Cell. and Comp. Physiol.* 25: 1, 1945.
5. Bang, F. B. *Proc. Soc. Exper. Biol. and Med.* 63: 5, 1946.
6. Bang, F. B. and R. M. Herriott. *Proc. Soc. Exper. Biol. and Med.* 52: 177, 1943.
7. Bawden, F. C. *Plant Viruses and Virus Diseases.* Leiden, Holland: Chronica Botanica Company, 1939.
8. Bawden, F. C., N. W. Pirie, J. D. Bernal and I. Fankuchen. *Nature* 138: 1051, 1936.
9. Beard, J. W. *Proc. Inst. Med.* 15: 294, 1945.
10. Beard, J. W. *J. Immunol.* 58: 49, 1948.
11. Beard, J. W., W. R. Bryan and R. W. G. Wyckoff. *J. Infect. Dis.* 65: 43, 1939.
12. Beard, J. W., H. Finkelstein and R. W. G. Wyckoff. *J. Immunol.* 35: 415, 1938.
13. Beard, J. W. and R. W. G. Wyckoff. *J. Biol. Chem.* 123: 461, 1938.
14. Beaudette, F. R. *Proc. Forty-Seventh Annual Meeting of the United States Live Stock Sanitary Association December 2, 3, 4,* 1943.
15. Bechhold, H. and M. Schlesinger. *Biochem. Z.* 236: 387, 1931.
16. Bechhold, H. and M. Schlesinger. *Z. Hyg. u. Infektionskrankh.* 112: 668, 1931.
17. Beijernick, M. W. *Zentr. Bakt. Parasitenk. Abt.* II. 5: 27, 1899.
18. Borrel, A. *Compt. rend. soc. biol.* 57: 642, 1904.
19. Boswell, F. W. *Brit. J. Exper. Path.* 28: 253, 1947.
20. Burnet, F. M. *Virus as Organism.* Cambridge, Mass.: Harvard University Press, 1945.
21. Burnet, F. M. and J. D. Ferry. *Brit. J. Exper. Path.* 15: 56, 1934.
22. Cohen, S. S. *J. Biol. Chem.* 156: 691, 1944.
23. Cohen, S. S. and T. F. Anderson. *J. Exper. Med.* 84: 511, 1946.
24. Cunha, R., M. L. Weil, D. Beard, A. R. Taylor, D. G. Sharp and J. W. Beard. *J. Immunol.* 55: 69, 1947.
25. Delbrück, M. *The Harvey Lectures* 41: 161, 1945–46.

26. DINGLE, J. H. *New Eng. J. Med.* 237: 845, 1947.
27. DOYLE, T. M. *J. Comp. Path. and Therap.* 40: 144, 1927.
28. DUGGAR, B. M. AND J. K. ARMSTRONG. *Ann. Missouri Bot. Gard.* 10: 191, 1923.
29. ELFORD, W. J. *J. Path. and Bact.* 34: 505, 1931.
30. ELFORD, W. J. *Brit. J. Exper. Path.* 17: 399, 1936.
31. ELFORD, W. J. AND C. H. ANDREWES. *Brit. J. Exper. Path.* 17: 422, 1936.
32. FINGELSTEIN, H., W. MARX, W. H. BRIDGERS AND J. W. BEARD. *Proc. Soc. Exper. Biol. and Med.* 39: 103, 1938.
33. FRANCIS, T., JR. *Science* 92: 405, 1940.
34. FRANCIS, T., JR. *The Harvey Lectures* 37: 69, 1941–42.
35. FRIEDEWALD, W. F. AND E. G. PICKELS. *Proc. Soc. Exper. Biol. and Med.* 52: 261, 1943.
36. FRIEDEWALD, W. F. AND E. G. PICKELS. *J. Exper. Med.* 79: 301, 1944.
37. GORTNER, R. A. *Science* 87: 529, 1938.
38. GREEN, R. G. *Science* 82: 443, 1935.
39. GREEN, R. H., T. F. ANDERSON AND J. E. SMADEL. *J. Exper. Med.* 75: 651, 1942.
40. GROUPÉ, V., J. OSKAY AND G. RAKE. *Proc. Soc. Exper. Biol. and Med.* 63: 477, 1946.
41. HOAGLAND, C. L. *Ann. Rev. Biochem.* 12: 615, 1943.
42. HOAGLAND, C. L., G. I. LAVIN, J. E. SMADEL AND T. M. RIVERS. *J. Exper. Med.* 72: 139, 1940.
43. HOAGLAND, C. L., J. E. SMADEL AND T. M. RIVERS. *J. Exper. Med.* 71: 737, 1940.
44. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL AND T. M. RIVERS. *Proc. Soc. Exper. Biol. and Med.* 45: 669, 1940.
45. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL AND T. M. RIVERS. *J. Exper. Med.* 72: 685, 1940.
46. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL AND T. M. RIVERS. *J. Exper. Med.* 74: 69, 1941.
47. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL AND T. M. RIVERS. *J. Exper. Med.* 74: 133, 1941.
48. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL AND T. M. RIVERS. *J. Exper. Med.* 76: 163, 1942.
49. HOOK, A. E., D. BEARD, A. R. TAYLOR, D. G. SHARP AND J. W. BEARD. *J. Biol. Chem.* 165: 241, 1946.
50. HUGHES, T. P., R. F. PARKER AND T. M. RIVERS. *J. Exper. Med.* 62: 349, 1935.
51. IWANOWSKI, D. *Bull. Acad. Imp. Sci. St. Petersburg. n.s.* 35: 67, 1892.
52. JENNINGS, H. S. *Science* 84: 445, 1936.
53. KALMANSON, G. AND J. BRONFENBRENNER. *J. Gen. Physiol.* 23: 203, 1939.
54. KNIGHT, C. A. *J. Exper. Med.* 79: 285, 1944.
55. *Ibid.* 80: 83, 1944.
56. *Ibid.* 83: 11, 1946.
57. *Ibid.* 83: 281, 1946.
58. *Ibid.* 85: 99, 1947.
59. *Ibid.* 86: 125, 1947.
60. KNIGHT, C. A. AND W. M. STANLEY. *J. Exper. Med.* 79: 291, 1944.
61. KRANEVELD, F. C. *Nederlandsch-Indische Bladen voor Diergeneeskunde* 38: 448, 1926.
62. LAUFFER, M. A. *Report of the New England Association of Chemistry Teachers 4*, 1941.
63. LAUFFER, M. A. *J. Biol. Chem.* 143: 99, 1942.
64. LAUFFER, M. A. *Arch. Biochem.* 13: 145, 1947.
65. LAUFFER, M. A. AND G. L. MILLER. *J. Exper. Med.* 80: 521, 1944.
66. LAUFFER, M. A. AND W. M. STANLEY. *J. Biol. Chem.* 135: 463, 1940.
67. LAUFFER, M. A. AND W. M. STANLEY. *J. Exper. Med.* 80: 531, 1944.
68. LURIA, S. E. AND T. F. ANDERSON. *Proc. Nat. Acad. Sci.* 28: 127, 1942.
69. LURIA, S. E., M. DELBRÜCK AND T. F. ANDERSON. *J. Bact.* 46: 57, 1943.
70. MACFARLANE, M. G. AND M. H. SALAMAN. *Brit. J. Exper. Path.* 19: 184, 1938.
71. MAGILL, T. P. *Proc. Soc. Exper. Biol. and Med.* 45: 162, 1940.
72. MARKHAM, R., K. M. SMITH AND D. LEA. *Parasitol.* 34: 315, 1942.
73. MCFARLANE, A. S. *Trans. Faraday Soc.* 36: 257, 1940.
74. MCFARLANE, A. S. AND M. G. MACFARLANE. *Nature* 144: 376, 1939.
75. MCFARLANE, A. S., M. G. MACFARLANE, C. R. AMIES AND G. H. EAGLES. *Brit. J. Exper. Path.* 20: 485, 1939.

76. McINTOSH, J. AND F. R. SELBIE. *Brit. J. Exper. Path.* 18: 162, 1937.
77. MEYER, K. F., C. M. HARING AND B. HOWITT. *Science* 74: 227, 1931.
78. MILLER, G. L. *J. Exper. Med.* 80: 507, 1944.
79. MILLER, G. L., M. A. LAUFFER AND W. M. STANLEY. *J. Exper. Med.* 80: 549, 1944.
80. MIRSKY, A. E. *Adv. Enzymol.* 3: 1, 1943.
81. MIRSKY, A. E. AND A. W. POLLISTER. *Proc. Nat. Acad. Sci.* 28: 344, 1942.
82. MOSLEY, V. M. AND R. W. G. WYCKOFF. *Nature* 157: 263, 1946.
83. MÜLLER, H. O. *Kolloid-Z* 99: 6, 1942.
84. NEURATH, H., G. R. COOPER, D. G. SHARP, A. R. TAYLOR, D. BEARD AND J. W. BEARD. *J. Biol. Chem.* 140: 293, 1941.
85. NORTHROP, J. H. *J. Gen. Physiol.* 21: 335, 1938.
86. NORTHROP, J. H. AND M. KUNITZ. *J. Gen. Physiol.* 7: 729, 1925.
87. PARKER, R. F. AND T. M. RIVERS. *J. Exper. Med.* 62: 65, 1935.
88. PAULING, L. *The Nature of the Chemical Bond* (2d ed.). Ithaca, New York: Cornell University Press, 1940.
89. PICKELS, E. G. AND J. E. SMADEL. *J. Exper. Med.* 68: 583, 1938.
90. PIRIE, N. W., in J. NEEDHAM AND D. E. GREEN. *Perspectives in Biochemistry.* Cambridge University Press, 1937. p. 11.
91. PIRIE, N. W. *Ann. Rev. Biochem.* 15: 573, 1946.
92. PRICE, W. C., R. C. WILLIAMS AND R. W. G. WYCKOFF. *Arch Biochem.* 9: 175, 1946.
93. RIVERS, T. M. *Bull. N. Y. Acad. Med.* 14: 383, 1938.
94. RIVERS, T. M. *Lane Medical Lectures: Viruses and Virus Diseases.* Stanford University Press, Stanford University, California: 1939.
95. RIVERS, T. M. *Science* 93: 143, 1941.
96. ROUS, P. *The Harvey Lectures* 31: 74, 1935.
97. RUFFILLI, B. *Biochem. Z.* 263: 63, 1933.
98. RUSKA, H. *Naturwissenschaften* 29: 367, 1941.
99. RUSKA, H. AND G. A. KAUSCHE. *Zentr. Bakt. Parasitenk Abt. I Orig.* 150: 311, 1943.
100. SCHLESINGER, M. *Z. Hyg. u. Infectionskrankh.* 114: 161, 1932.
101. SCHLESINGER, M. *Biochem. Z.* 264: 6, 1933.
102. SCHLESINGER, M. *Biochem. Z.* 273: 306, 1934.
103. SCHLESINGER, M. *Nature* 138: 508, 1936.
104. SCHMIDT, G. AND S. J. THANNHAUSER. *J. Biol. Chem.* 161: 83, 1945.
105. SEVAG, M. G., J. SMOLENS AND D. B. LACKMAN. *J. Biol. Chem.* 134: 523, 1940.
106. SHARP, D. G. *Unpublished work.*
107. SHARP, D. G., M. H. HEBB, A. R. TAYLOR AND J. W. BEARD. *J. Biol. Chem.* 142: 217, 1942.
108. SHARP, D. G., A. E. HOOK, A. R. TAYLOR, D. BEARD AND J. W. BEARD. *J. Biol. Chem.* 165: 259, 1946.
109. SHARP, D. G., A. R. TAYLOR, D. BEARD AND J. W. BEARD. *Proc. Soc. Exper. Biol. and Med.* 50: 205, 1942.
110. SHARP, D. G., A. R. TAYLOR, D. BEARD AND J. W. BEARD. *J. Biol. Chem.* 142: 193, 1942.
111. SHARP, D. G., A. R. TAYLOR, D. BEARD AND J. W. BEARD. *Arch. Path.* 36: 167, 1943.
112. SHARP, D. G., A. R. TAYLOR AND J. W. BEARD. *J. Biol. Chem.* 163: 289, 1946.
113. SHARP, D. G., A. R. TAYLOR, A. E. HOOK AND J. W. BEARD. *Proc. Soc. Exper. Biol. and Med.* 61: 259, 1946.
114. SHARP, D. G., A. R. TAYLOR, I. W. MCLEAN, JR., D. BEARD AND J. W. BEARD. *J. Biol. Chem.* 156: 585, 1944.
115. SHARP, D. G., A. R. TAYLOR, I. W. MCLEAN, JR., D. BEARD AND J. W. BEARD. *J. Biol. Chem.* 159: 29, 1945.
116. SHARP, D. G., A. R. TAYLOR, I. W. MCLEAN, JR., D. BEARD, J. W. BEARD, A. E. FELLER AND J. H. DINGLE. *J. Immunol.* 48: 129, 1944.
117. SHEDLOVSKY, T. AND J. E. SMADEL. *J. Exper. Med.* 72: 511, 1940.
118. SHOPE, R. E. *J. Exper. Med.* 54: 373, 1931.
119. SHOPE, R. E. *J. Exper. Med.* 58: 607, 1933.

120. SHOPE, R. E. *The Harvey Lectures* 31: 183, 1935–36.
121. SMADEL, J. E. AND C. L. HOAGLAND. *Bact. Rev.* 6: 79, 1942.
122. SMADEL, J. E., E. G. PICKELS AND T. SHEDLOVSKY. *J. Exper. Med.* 68: 607, 1938.
123. SMADEL, J. E., E. G. PICKELS, T. SHEDLOVSKY AND T. M. RIVERS. *J. Exper. Med.* 72: 523, 1940.
124. SMITH, W., C. H. ANDREWES AND P. P. LAIDLAW. *Lancet.* 2: 66, 1933.
125. STANLEY, W. M. *Science* 81: 644, 1935.
126. STANLEY, W. M. in R. DOERR AND C. HALLAUER. *Handbuch der Virusforschung.* Wien: Verlag von Julius Springer, 1939.
127. STANLEY, W. M. *J. Biol. Chem.* 129: 405, 1939.
128. STANLEY, W. M. *Physiol. Rev.* 19: 524, 1939.
129. STANLEY, W. M. *Ann. Rev. Biochem.* 9: 545, 1940.
130. STANLEY, W. M. *J. Exper. Med.* 79: 255, 1944.
131. STANLEY, W. M. *J. Exper. Med.* 79: 267, 1944.
132. TAYLOR, A. R. *Unpublished work.*
133. TAYLOR, A. R. *J. Biol. Chem.* 153: 675, 1944.
134. TAYLOR, A. R. *J. Biol. Chem.* 165: 271, 1946.
135. TAYLOR, A. R., D. BEARD, D. G. SHARP AND J. W. BEARD. *J. Infect. Dis.* 71: 110, 1942.
136. TAYLOR, A. R., D. G. SHARP, D. BEARD AND J. W. BEARD. *J. Infect. Dis.* 72: 31, 1943.
137. TAYLOR, A. R., D. G. SHARP, D. BEARD, J. W. BEARD, J. H. DINGLE AND A. E. FELLER. *J. Immunol.* 47: 261, 1943.
138. TAYLOR, A. R., D. G. SHARP AND J. W. BEARD. *J. Infect. Dis.* 67: 59, 1940.
139. TAYLOR, A. R., D. G. SHARP, I. W. MCLEAN, JR., D. BEARD, J. W. BEARD, J. H. DINGLE AND A. E. FELLER. *J. Immunol.* 48: 361, 1944.
140. TENBROECK, C. AND M. H. MERRILL. *Proc. Soc. Exper. Biol. and Med.* 31: 217, 1933.
141. WILLIAMS, R. C. AND R. W. G. WYCKOFF. *Proc. Soc. Exper. Biol. and Med.* 58: 265, 1945.
142. WOODRUFF, C. E. AND E. W. GOODPASTURE. *Am. J. Path.* 5: 1, 1929.
143. WOODRUFF, C. E. AND E. W. GOODPASTURE. *Am. J. Path.* 6: 713, 1930.
144. WYCKOFF, R. W. G. *Proc. Soc. Exper. Biol. and Med.* 36: 771, 1937.
145. WYCKOFF, R. W. G. *J. Gen. Physiol.* 21: 367, 1938.
146. WYCKOFF, R. W. G. *Science* 101: 129, 1945.
147. WYCKOFF, R. W. G. *Nature* 157: 764, 1946.
148. WYCKOFF, R. W. G. *Science* 104: 21, 1946.
149. WYCKOFF, R. W. G. *Proc. Soc. Exper. Biol. and Med.* 66: 42, 1947.

THE RELATION OF NUTRITIONAL DEFICIENCIES TO GRAYING

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THE LITERATURE on the physiology, morphology and pigmentation of hair has been reviewed by Bloch (1), Danforth (2) and Urbach (3). Although considerable work remains to be done to clarify these fields of knowledge the following points appear to be fairly well established.

The color of hair is normally caused by the deposition of pigment (melanin) into the hair shaft from the follicle during growth of the hair. Different colors and shades are caused by differences in the color of the pigment deposits and also in the way in which the pigment is diffused or concentrated in granules. White hair contains no pigment. Graying of normally pigmented hair is apparently caused by a failure of the hair follicles to deposit pigment in the hair at the time the hair is being formed.

Pigmentation of feathers has been extensively studied by Willier (4). The chick embryo provides a rather ideal medium for study of the relation of genetic factors to pigmentation. Willier has concluded that the ability to form pigment exists only in certain cells, the so-called melanophore cells. These cells arise from the neural crest and migrate into the skin and its derivatives—the hair and feather follicles. In the absence of melanophores the hair and feather structure is otherwise normal but without pigment. When melanophore cells were removed from the neural crest of embryos of Barred Plymouth Rock chicks and grafted to wing buds of White Leghorn embryos, the White Leghorn chicks developed the pigmented wing feathering characteristic of Barred Plymouth Rocks.

According to Willier the specific color or pattern produced in feathers depends on the genetic constitution of the melanophores and partly on the physiology of the feather follicle, i.e., its growth rate etc. Experiments were cited which indicate that the melanophore cells which arise from the neural crest migrate laterally and invade the skin, the hair and feather follicles and furnish melanin to these structures. This is true both in mammals and in birds and is thought to account for the characteristic coloring of animals in general.

Graying, depigmentation or failure of pigmentation of hair has been variously referred to as 'achromotrichia' (5), 'canities' (6) and 'achromachia' (7). The word achromotrichia, or simply graying, will be used throughout this review to describe the condition of 'absence of pigment from hair or feathers'. These terms have received widespread use in recent scientific literature. There appears to be general agreement among nutritionists that graying of fur or feathers may be brought about in many species of animal, usually during the stage of growth, by imposing deficiencies of certain specific mineral elements or factors of the vitamin B-complex. Various names have been used in referring to these pigmentogenic nutritional factors normally

present in foods, such as trichochromogenic (8), anti-gray hair, anti-achromotrichia and chromotrichia (9) factors. For purposes of this review the various nutritional factors which appear to be needed for normal pigmentation processes are referred to in general as pigmentation factors, or as factors necessary for pigmentation of hair, feathers or skin.

Sufficient experimental evidence is not at hand to evaluate all of the various possible causes of graying in man. Barahal (10) has cited instances to indicate that extreme psychotic and psychoneurotic states or cerebral trauma may predispose to rapid and premature graying. He lists as possible causes of gradual premature graying the following: heredity, emotional strain, endocrine disturbance, dietary deficiency and the chronic diseases—tuberculosis, syphilis, malaria, diabetes, Addison's disease and Simmond's disease. He further reviews the possibilities advanced by others that rapid graying of hair may in some instances be associated with physical changes of the hair, such as thickening and opacity of the outer layer and the presence of air bubbles in the hair itself. It should be noted that as early as 1910 Stieda (11) discredited many of the famous instances of literature and history which tell of sudden graying. The American Medical Association (12) likewise early endorsed the viewpoint that sudden graying of hair is a virtual impossibility.

The finding that hair and feather color can be greatly influenced by nutritional controls in growing experimental animals has led to much speculation with regard to the possibility of arresting or correcting graying in humans. On purely theoretical grounds Hrdlicka (13) repudiated the idea that the influence of essential nutrients or drugs can have more than a temporary stimulating effect on the normal pigmentation processes. He stated empirically that graying is a normal symptom of the aging process and is an outward symptom of declining metabolism. Gibbs (9), however, pointed out that seasonal changes in the color of a variety of animals is a normal process not related to age and that there are many reasons to assume that the pigmentation processes are subject to drug action like most other body functions. The two viewpoints are not in strict opposition and the interesting question whether graying can be delayed in the aging human by nutritional or other means has yet to be answered.

Although this review deals chiefly with reports concerning nutritional studies, there is included a brief discussion of the chemistry of pigment formation, and an attempt is made to correlate some of the facts in a general way. Brief reviews on various phases of the problem have appeared from time to time in *Nutrition Reviews* (14).

EARLY EXPERIMENTAL OBSERVATIONS

Although Stieda (11) predicted it in 1910 the observation that deficiency in diet may predispose to graying was not made until 1922 when Niemes and Wacker (15) reported graying in black rats fed milk and starch. The graying was cured in a few weeks when the rats were returned to a stock diet. At about the same time Hartwell (16) in England observed fawning in rats on summer milk but not on winter milk when they grew less rapidly. She suspected a protein deficiency, particularly deficiency of tyrosine and tryptophan and reported the curative effect of adding 'food

casein' to the bread and milk diet. Hartwell made the interesting observation that there must be a small store at birth of pigment-forming essentials since only one suckling rat out of about a thousand litters developed a gray coat on first growth of fur.

Bakke, Aschehoug and Zbinden (17) in 1930 described graying of rats on synthetic diets containing wheat germ as a source of the vitamin B-complex. Since the use of whole wheat in place of wheat germ did not cause graying, these authors concluded that wheat germ probably contains a toxic factor which predisposes to unnatural graying. They apparently let the matter drop at that.

In 1931 Keil and Nelson (18) in studying the effects of copper deficiency produced by feeding milk diets reported the interesting fact that copper deficient rats become depigmented. When copper was supplied there was a gradual return to normal hair color. In 1934 Gorter (19) at Delft, Holland, produced graying in cats, rabbits and rats on a multiplicity of diets. From his extensive experiments Gorter concluded that copper was the chief, if not the only, active principle in the curative substances he examined. Gorter's work is difficult to assess in light of recent findings because the diets he used were varied and ill-defined. Sjollema (20) in Denmark reported in 1938 the concomitant occurrence of anemia and depigmentation in cattle on copper deficient pasture.

Zinc, also, appears to be concerned in the mechanism of pigmentation. Stirn, Elvehjem and Hart (21) found that acute zinc deficiency caused retardation of growth and depigmentation in young albino rats. Both deficiency symptoms were rapidly alleviated by administration of minute quantities of soluble zinc salts. The fact that zinc has been found in considerable amount in hair (22) is interesting in this regard, although little is known of the mechanism of its action.

FACTORS OF THE VITAMIN B-COMPLEX

That organic as well as inorganic nutrients are involved in graying has been shown by a large number of workers but was specifically referred to by Free (23) who demonstrated the inefficacy of B-complex factors to cure copper deficiency achromotrichia in rats and the equal inefficacy of copper to cure achromotrichia induced by B-complex deficiency.

Widespread interest in the problem from the nutritional side did not come, however, until Morgan, Cook and Davison (24) and Lunde and Kringstad (25) in 1938 reported graying in rats maintained on synthetic diets deficient only in the filtrate factors of the vitamin B-complex, i.e., factors other than thiamin, riboflavin and pyridoxine, and not adsorbed on Fuller's earth. Synthetic diets with casein, sucrose, salts, corn oil or other unsaturated fat and cod liver oil plus thiamin, riboflavin and pyridoxine induced graying in black and piebald rats in six to seven weeks. Animals on such diets, however, showed various gross deficiency symptoms, all of which were curable by addition of crude filtrate fractions. Both groups (26, 27) made the pertinent observation that yeast and yeast extracts always promoted good growth, but did not always cure graying. Likewise wheat germ and cane molasses induced rapid growth, but only late and incomplete cure of grayness, while rice bran and liver filtrates had less effect on growth but caused prompt darkening of the fur. Morgan

and Simms (27) produced nutritional achromotrichia in rats, dogs and guinea pigs on similar diets deficient in the 'filtrate fraction' of the vitamin B-complex. Both of the above groups (28, 29) also reported achromotrichia in the silver fox on diets designed to be specifically deficient in the so-called 'anti-gray hair vitamins'. Lunde and Kringstad (30) stated that they could distinguish the anti-graying factor from the growth factor by the slightly greater instability to heat of the former.

Oleson, Elvehjem and Hart (5) reported in 1939 that pantothenic acid concentrates were ineffective in preventing nutritional achromotrichia. Early in 1940, however, György, Poling and Subbarow (32) indicated the curative effect of pantothenic acid in concentrates of that vitamin, but concluded that it is not the only factor involved. Later György and Poling (33) reported practically complete cure of graying in 5 to 7 weeks with daily doses of 75–100 μg. of synthetic d(+)calcium pantothenate. Still later (34) they showed some effect for biotin as well, but stated that even with biotin plus pantothenic acid, the pigmentation was still not entirely normal.

Nielsen, Oleson and Elvehjem (35) announced in 1940 that they had succeeded in isolating a highly active crystalline chromotrichial substance of unknown nature. In the same year Unna and Sampson (36) claimed clear-cut cure of graying in rats on synthetic diet with calcium pantothenate at 100 μg. per day. Considerable evidence was forthcoming, however, that pantothenic acid is only one, although it is probably the most important, of the filtrate factors needed for normal pigmentation. Dimick and Lepp (37) indicated the presence of additional chromotrichial factors in rice bran extracts and Williams (38) reported the inadequacy of pantothenic acid alone to cure graying. Frost, Dann and Moore (39) showed that the curative capacity of liver and yeast extracts is not entirely dependent on their pantothenic acid content, but that liver fractions containing relatively small amounts of pantothenic acid may be curative. They postulated the presence of an additional factor in yeast and liver which appeared to potentiate the action of pure pantothenic acid.

Early in 1941, Ansbacher (40) announced that p-aminobenzoic acid is a vitamin, and an achromotrichia factor as well. He reported that rats became gray on his ration supplying a high level of all of the known B-complex factors and that blackening occurred in one month following administration of 3 mg. daily of p-aminobenzoic acid. Later Martin and Ansbacher (41) reported cure of achromotrichia induced by hydroquinone toxicity in mice. In these experiments 0.8 mg. daily of p-aminobenzoic acid was reported to cure the graying more effectively than rice bran concentrates. Emerson (42) and Unna, Richards and Sampson (43) soon reported negative results with p-aminobenzoic acid alone or in combination with pantothenic acid and were unable to produce graying in rats with a diet similar to the one Ansbacher had described. Experiments at Wisconsin (44) and in this laboratory (45) also were entirely negative with p-aminobenzoic acid. The literature on p-aminobenzoic acid has been reviewed by Ansbacher (46).

The complexity of the graying and pigmentation mechanisms assumed even greater proportions in 1941 with the findings that dietary adequacy of cystine, choline and sodium chloride may be involved together with the fore-mentioned compounds. Insufficiency of cystine supply in the ordinary synthetic diet containing 18 per cent

of washed casein was indicated by Pavcek and Baum (47) who reported that administration of 70 mg. daily of cystine to grayed rats augmented the curative effect of calcium pantothenate and stimulated growth above that on pantothenate alone. Owens, Trautman and Woods (48) reported that choline deficiency results in the rusting of fur in albino rats, as does deficiency of pantothenic acid, and suggested that this may have some bearing on the achromotrichia problem. In this regard the effect of inositol on hair growth in mice (49) and in rats (50) should not be overlooked, although no evidence has yet been forthcoming that this factor has to do with pigmentation. Ralli, Clarke and Kennedy (51) reported that the amount of salt added to the filtrate factor-deficient diet influenced the incidence, extent and degree of graying in rats. Rats on low salt intake grayed 55 days sooner than those on high salt intake. Changes were also seen in the adrenals of these animals. This finding added to the already strong evidence that adrenal function is concerned with pigmentation.

Following the more or less general agreement among nutritionists that p-aminobenzoic acid was not concerned to a prominent degree in the normal pigmentation of experimental animals (42–44) came the report of McGinnis, Norris and Heuser (52) that an unidentified factor in brewer's yeast, other than pantothenic acid, is necessary for the normal feathering and pigmentation of Rhode Island Red chicks. Martin (53) and Wright and Welch (54) produced some achromotrichia in rats fed sulfadiazine or succinylsulfathiazole and reversed the condition by feeding concentrates known to contain folic acid. Frost and Dann (55) were able to produce a high incidence of graying in dogs raised on highly purified diets with adequate calcium pantothenate, copper, zinc, biotin, inositol, choline, cystine and p-aminobenzoic acid. The achromotrichia was associated with anemia, and both conditions regressed with liver extract or yeast feeding. Early in 1946 Frost, Dann and McIntire (56) reported the prevention of graying in Black-Leghorn chicks which received 10 μg. per day of synthetic pteroylglutamic acid (folic acid) in addition to a diet containing all other known dietary factors. Pigmentation was comparable to that seen in chicks which received a 10 per cent yeast supplement. Graying was pronounced in chicks which received only 5 μg. pteroylglutamic acid by injection per day. Below this level feathering and growth were very poor. Lillie and Briggs (57) confirmed the prevention of depigmentation in the chick by the addition of synthetic pteroylglutamic acid to purified diets adequate in all other known factors.

A possible interrelationship between pantothenic acid and pteroylglutamic acid in metabolism is indicated by the work of Wright and Welch (54). Their work showed that liver storage of pantothenic acid was favorably influenced by the simultaneous administration of folic acid-rich concentrates and biotin to succinylsulfathiazole fed rats. From this one might deduce that utilization of pantothenic acid is in some way dependent on the presence of pteroylglutamic acid. Other work (37, 39) had previously indicated that low levels of pantothenic acid as supplied in yeast and liver were more effective in the prevention of achromotrichia in growing rats than was a four-fold level of pure d(+)calcium pantothenate. The inference was that unknown factors in the natural materials were acting synergistically with pantothenic acid in the prevention of graying.

In the early work on the effect of pantothenate on graying considerable discrepancy was encountered between results in different laboratories. Complete prevention or cure (43, 44), incomplete prevention or cure (34, 37, 39, 40, 31, 58, 59), and no prevention or cure (38) of achromotrichia in rats or chicks by pantothenate were reported in 1940–41. It is probable that the different diets used contained different levels of folic acid. There is the further likelihood that intestinal synthesis of folic acid may proceed at different rates under differing conditions. That the rat requires folic acid when sulfaguanidine or succinylsulfathiazole is fed and that this artificial deficiency state is accompanied by achromotrichia has been demonstrated (53, 54). The rat can dispense with pteroylglutamic acid even on highly synthetic diets; however, the needs for growth and all phases of metabolism may not always be completely met by intestinal synthesis of this factor. Under these conditions transitory graying may occur.

Spontaneous cure of graying in rats approaching maturity has been reported by Unna and Richards (60) and Schwarz (61) in rats receiving minimal levels of pantothenate. Unna and Richards (60) reported a decrease in the pantothenate requirement of rats from 100 μg. per day at 3 weeks to 25 μg. per day at 10 weeks of age. Schwarz (61) made a quantitative study of the effect of low levels of pantothenic acid with regard to pigmentation in relation to growth. He reported that weanling piebald rats fed 29 micrograms of d(+)pantothenic acid per day gained an average of 11 grams per week and only 7 per cent of them showed graying. A similar group which received 5 micrograms of d(+)pantothenic acid per day gained 9.5 grams weight and 100 per cent of the animals became gray. Schwarz concluded that there is competition within the animal for pantothenic acid for the unrelated functions of growth and melanin formation and that the needs for growth are generally first satisfied. On low prophylactic doses graying appeared before growth decreased. In studying the effect of pantothenic acid on growth Schwarz (62) noted that weanling rats made pantothenate deficient by a standardized method showed a weight response in close proportion to the size of the dose. Thus each microgram of d(+)pantothenic acid gave about 62.5 milligrams increase in body weight over a wide dosage range. The effect of sub-optimal levels of pantothenic acid to satisfy certain physiological functions without meeting the needs for growth is further seen in the work of Mills, Shaw, Elvehjem and Phillips (63). These workers indicated that only 4 to 25 micrograms of pantothenic acid per day was sufficient to prevent the appearance of adrenal necrosis from developing in weanling rats.

The possible effects of intestinal synthesis of pantothenic acid and folic acid on achromotrichia are difficult to define. Although there has been no general verification of the importance of p-aminobenzoic acid in animal nutrition, there have been some indications (53, 64) that p-aminobenzoic acid acts indirectly by stimulating microbial synthesis of other factors which may then become available to the animal. The chromotrichial effects reported for biotin (34, 65) may be related to some extent to a reduced synthesis of this factor in the intestinal tract (66, 67) under certain conditions. The clearcut graying produced by Emerson and Keresztesy (65) in rats fed 15 per cent dried eggwhite in the diet is, however, more clearly related to formation of the unavailable avidin-biotin complex than to a failure of intestinal synthesis.

A still further deficiency effect related to abnormal feather pigmentation in bronze poults has been described by Fritz *et al.* (68). The syndrome was produced on diets containing a high percentage of vegetable proteins and was prevented by adding lysine. The indication was that 1.1 to 1.2 per cent lysine in the diet is needed for normal feather pigmentation and optimum growth in poults.

The interesting point has been advanced by Morgan and Simms (27) that abundance of nicotinic acid and pyridoxine in the diet predisposes to rapid graying in animals on synthetic diet. This type of diet leads also to adrenal and thyroid damage and to unnatural thymus survival. According to Morgan the changes which occur in the glands of animals deficient in pigmentation factors are typical of senescence. Further studies relative to this type of observation and the possible import in human nutrition are needed.

TABLE 1. DIETARY FACTORS REPORTED TO BE NECESSARY FOR NORMAL PIGMENTATION IN ANIMALS AND BIRDS

DIETARY FACTOR	REPORTED BY	YEAR FIRST REPORTED	SPECIES AND REFERENCE NO.
——	Niemes and Wacker	1922	Rat (15)
——	Hartwell	1923	Rat (16)
Copper	Keil and Nelson	1931	Rat (18, 23); cats and rabbits (19); cattle (20)
Zinc	Stirn, Elvehjem and Hart	1935	Rat (21)
Filtrate factor	Morgan, Cook and Davison	1938	Rat (24–30); dog (27, 55); fox (28); guinea pig (27)
Pantothenic acid	György and Poling	1940	Rat (32–39, 58–59); chick (31, 52); mouse (34)
P-aminobenzoic acid	Ansbacher	1941	Rat (40); mouse (41)
Folic acid (concentrate)	Martin	1942	Rat (53, 54)
Pteroylglutamic acid	Frost, Dann and McIntire	1946	Chick (56)
Lysine	Fritz, Hooper, Halpin and Moore	1946	Turkey (68)

In table 1 is presented a brief summary of the dietary factors thus far reported to be necessary for normal pigmentation of various animals and birds, together with the initial investigators, the year reported, the species studied and some of the key references.

OBSERVATIONS IN HUMAN BEINGS

A few attempts to cure graying in humans have been scientifically reported, and several reports have appeared in the popular press and in trade journals. Some of the latter have been referred to by Brandaleone, Main and Steele (69). Only those papers which have appeared in recognized scientific journals will be reviewed here.

In 1941 after p-aminobenzoic acid had been reported to be an achromotrichia factor in rats (40), Sieve reported (70, 71) changes in hair color after three to eight weeks in humans given large doses of p-aminobenzoic acid, i.e., about 100 mg. twice daily by mouth. Experimental details and observations were not clearly described in these reports.

In 1943 Kerlan and Herwick (72) reported a clinical study in 27 white men and women with graying hair given 20 mg. of calcium pantothenate daily. No significant change in hair color was noted and it was concluded that calcium pantothenate alone is of no value in the restoration of color to gray hair in humans. Similar negative results were reported the same year by Vorhaus, Gompertz, and Feder (73). Brandaleone, Main and Steele (74) also in 1943 reported that 2 out of 19 elderly grayed individuals treated daily for eight months with 100 mg. calcium pantothenate, 200 mg. para-aminobenzoic acid and about 50 grams brewer's yeast showed a significant change toward a return of normal hair color. In 1944 these same authors (69) reported their previous studies in greater detail and extended the study to include 14 younger individuals. None of the younger individuals showed a decrease in grayness with different combinations of calcium pantothenate, para-aminobenzoic acid, and brewer's yeast or yeast concentrate.

The authors point out that the hair of the two individuals who showed a positive effect gradually became grayer again after medication was stopped. The fact that both of these individuals received brewer's yeast as well as calcium pantothenate and para-aminobenzoic acid may be of some significance, especially in view of the more recent animal work indicating that folic acid, and possibly other factors in yeast and liver extract, play a part in normal pigmentation in chicks and dogs.

The appearance of graying in severely avitaminotic children has been observed in various parts of the world. Nicholls (75) reported that children in Malaya who live largely on manioc root exhibit symptoms of multiple nutritional deficiencies, one of the symptoms being graying of the hair. When the children were fed a good diet the proximal parts of the hair grew in normally pigmented, while the distal part remained gray. Graying was also seen in infants weaned on cereal gruels. A considerable loss of pigment was rarely seen and no change in the texture of the hair was noted.

Recently, nutritional deficiencies in children of a severity uncommon in the United States have been extensively observed also by Chavarria *et al.* (76) in Costa Rica. These authors reported that depigmentation of the hair is a rather generalized symptom in children severely deprived of various nutritional factors, where the diagnostic symptoms are those of pellagra, beri-beri, riboflavin deficiency or vitamin A deficiency. Undoubtedly these children suffer from a combination of many nutritional deficiencies and it is not likely that therapy with any one nutritional factor will effect a complete cure. It was reported that biotin, 0.25 mg. two or three times daily, appeared to accelerate the cure of the nutritional alopecia and achromotrichia over that observed on the hospital diet alone, or with added thiamin and nicotinamide. Because the curative experiments were not controlled definite conclusions could not be drawn as to the effect of individual vitamins. It was clear, however, that the depigmentation was easily reversible by adequate diet in those children who survived this severe vitamin deficiency state.

Williams (77) in 1933 in South Africa was among the first to observe the changes in hair color associated with severe nutritional deficiencies in children on maize diets. Trowell later (78) suggested that the disease was infantile pellagra. Gillman and Gillman (79) in 1945 stated that the disease, which they accept provisionally as pellagra, is prevalent among the native children of South Africa. The latter workers,

in a careful review of the symptoms and treatment of the disease, state that nicotinic acid and other crystalline vitamins are ineffective and even appear to be contra-indicated in cases of severe infantile pellagra. The severity of the deficiency is indicated by a record of 40 to 60 per cent mortality, despite the administration of the then known vitamins and a full diet. It was noted that if the liver had not been severely damaged and if the children took their feedings they would recover slowly on any form of rational therapy. The greatest difficulty was the complete loss of appetite and inanition. Results with injectable liver extract and powdered stomach were far superior to those with vitamins. Powdered stomach, especially, was considered a life-saving drug in the treatment of the disease.

The fact that the above-mentioned nutritional deficiency disease is entirely resistant to nicotinic acid and is cured by natural materials known to contain folic acid and other yet unknown factors suggests further experimental approaches to the disease. For instance, it would be of significance to determine whether folic acid alone, and in combination with other vitamin B-complex factors, will cure the disease. For purposes of this review it would be especially interesting to know whether folic acid together with other known pigmentation factors would reverse the graying which apparently occurs as a usual symptom of the disease. The relationship between folic acid and the anti-pernicious anemia fraction of liver extract, and the relationship between the latter factor and dried stomach, are at present diffuse but many indications suggest that there is a rather close biochemical relationship between them.

Gillman and Gillman (80) very appropriately quote the suggestion made to them by personal communication by Trowell that the name for the disease, infantile pellagra, originally proposed by him be replaced by the term 'malignant malnutrition'.

ENDOCRINE FACTORS

Certain other investigations relating to the achromotrichia problem may be reviewed here. The fact that acute pantothenic acid deficiency results in adrenal damage and the fact that adrenalectomy stimulates hair growth (81) suggested the use of cortical hormones for prevention of graying. Mushett and Unna in 1941 (82) reported the ineffectiveness of cortin, desoxycorticosterone, thyroid extract, U.S.P., and anterior pituitary extract to replace pantothenic acid for prevention of graying or adrenal hemorrhage in rats on pantothenic acid deficient diets. The preparations assayed both curatively and prophylactically were without effect.

That the adrenals are in some way involved in pigmentation processes is clearly indicated from the work of Ralli and her associates. In 1941 Ralli, Clarke and Kennedy (51) reported that the amount of salt added to filtrate-factor deficient diets influenced the incidence, extent and degree of graying in rats. Rats on low salt intake became gray 55 days sooner than those on high salt intake. Changes were seen in the adrenals of all animals. In 1943 Ralli and Graef (83) discovered that adrenalectomy causes a marked transitory hyperplasia of the hair bulbs and follicles and increased hair growth and deposition of melanin both in normal and in pantothenic acid deficient rats. The effect was less marked in the deficient animals than in the normal controls. It was noted that pantothenic acid deficient animals lose skin pigment as well as hair pigment and that adrenalectomy stimulates the return

of pigment to both skin and hair. Histologic studies indicated that the graying of hair was associated with some change in the hair follicles and a cessation of melanin deposition. Ralli and Graef (84) later showed that injection of desoxycorticosterone acetate following adrenalectomy in large measure inhibited the increase in melanin deposition and hair growth both in normal and in pantothenic acid deficient animals. These experiments suggest involvement of the corticosterone hormones in the overall mechanism of pigmentation. Ralli and Graef (85) reported graying in 284 rats on a filtrate factor deficient diet, but only a minor incidence of adrenal damage. The graying was most pronounced in rats on low salt intake and in rats receiving extra nicotinic acid.

Willier (4) cites experiments which indicate that melanophores from chick embryos respond in a specific fashion to various hormones, such as the sex hormones, thyroxin and desoxycorticosterone. Such experiments furnish a basis for studying the wide differences in pigmentation between different species of birds, and between the two sexes in the same species.

The effect of sex in mice on purified diets deficient in certain of the factors of the vitamin B-complex has been studied by Gerstl and Goldfarb (86). The incidence of graying was reported to be much higher in males than in females and the conclusion was drawn that in the strain of mice used hormonal factors play a part. Forbes (87) reported that estrogen implants caused local pigmentation of fur, but that testosterone propionate failed to do so.

CHEMISTRY OF PIGMENTATION

Melanin, the insoluble pigment of skin and hair, is amorphous and has not been well characterized chemically; however, the beginning steps leading to its final formation in plants and animals seem fairly well understood. Production of melanin may be observed in the blackening of cut potatoes on exposure to air or in the rapid blackening of catechol or tyrosine in the presence of certain plant extracts. Tyrosine is thought by Rothman (88) to be the unique beginning material for melanin formation both in plants and animals. *In vitro* experiments have shown that tyramine and adrenaline for which tyrosine is a precursor will yield pigments indistinguishable from melanin in presence of the enzyme tyrosinase.

The scheme on page 378 for melanin formation was postulated by Bloch (1) and studied in detail by Raper (89). The final agglomeration of the water-insoluble melanin granules which are deposited in the skin and hair by the melanoblast cells is not well understood.

The presence of a specific enzyme, dopa oxidase, in melanoblast pigmentation was postulated in 1917 by Bloch (90), but its actual existence was not proved. According to the work of Figge (91) tyrosinase may catalyze both the oxidation of tyrosine to dopa and the oxidation of dopa to dopa quinone. The second reaction is thought to be inhibited by the presence in excess of certain compounds having a low oxidation-reduction potential. Ascorbic acid and glutathione in excessive amounts hinder the forward progress of this reaction and may even reverse it. Only the first two steps in the above scheme are thought to require the intermediation of an enzyme since the remaining reactions proceed spontaneously. Arnow (92) has

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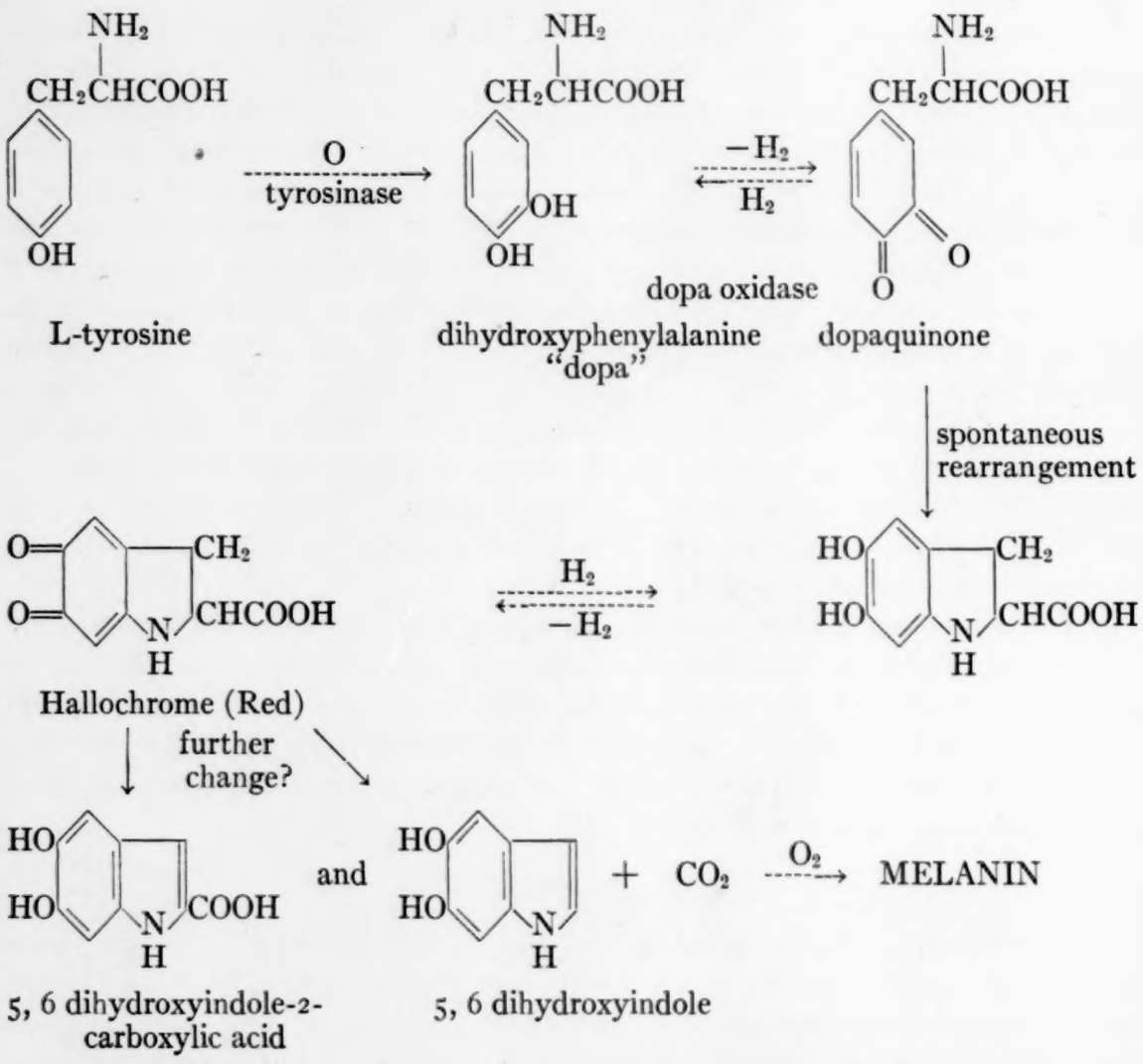

shown that tyrosine can be converted to dopa by irradiation with ultraviolet light, and this may occur to some extent *in vivo*. Rothman (93, 94) has further elaborated the effect of ultraviolet light in production of melanin and has demonstrated the catalytic effects of both ferrous salts and ascorbic acid. *In vitro* experiments were reported which indicated a close similarity in melanin formation from tyrosine by tyrosinase and by actinic stimulation. Ascorbic acid was shown to stimulate melanin formation but to reduce preformed melanin to lighter colored products, both *in vitro* and *in vivo*.

Demonstration of the presence and functional significance of phenol oxidases in mammalian tissue has generally proved difficult and neither tyrosinase nor dopa oxidase has been isolated from normal skin. Bloch (1) in 1917 reported experiments wherein dihydroxyphenylalanine was incubated with skin and over a period of time melanin was formed. The microscopic dopa reaction described by Bloch as a specific enzymatic process was used in early work by him as an indicator of the potential capacity of pigment formation in single cells. Hogeboom and Adams (95) reported the presence of a phenolase in a mouse melanoma which oxidized both tyrosine and dihydroxyphenylalanine to melanin and suggested that this is probably a property of normal tissue, though in much smaller concentration. Although the experiments

were not always successful, Onslow (96) and Pugh (97) have confirmed the 'dopa' reaction in skin. According to Rothman (93), Bloch's theory of the dopa reaction as a specific enzymatic process is in accord with all facts in the physiology and pathology of the pigmentation process.

Richter and Clisby (98) made the interesting observation that phenylthiocarbamide, a compound which inhibits the copper containing oxidative enzymes of tissue, causes graying in one to two months when fed to black rats in amounts of 1 to 14 mg. per rat day. When the phenylthiocarbamide feeding was stopped the hair again became black in about three months. In light of the above work Bernheim and Bernheim (99) studied the effect of phenylthiocarbamide on the tyrosinase of potatoes and mushrooms, and on the oxygen uptake of rat tissue slices and cell suspensions. The compound was found to be a very effective inhibitor of tyrosinase, but relatively ineffective against various tissue enzymes. Although the toxic effects of phenylthiocarbamide in the rat cannot be explained by its action on tyrosinase, it appears highly probable that the compound causes graying by inhibition of a specific copper-protein enzyme system in the melanoblasts of the skin, the hypothetical dopa oxidase.

An explanation of a few of the forementioned nutritional effects can be attempted in light of the above chemical scheme. Since copper is known to be the prosthetic group of all of the various phenol and polyphenol oxidases (100–102, 95), a deficiency of this element might conceivably lead to an interruption of the production of melanin at its very beginning. The depigmentation of hydroquinone poisoning which has been studied in rats and cats by Oettel (103) may result from accumulation of hydroquinone in the melanoblasts, where its reducing effect might be expected to interfere with either of the reversible reactions shown in the scheme. The hyperpigmentation of Addison's disease has been alleviated with vitamin C (104) which is known to act toward reversal of the above scheme. Goodman (105) has reported initial stimulation of pigment formation in two patients with alopecia areata by topical application of anthralin (dihydroxyanthranol), a reducing compound. Persistent use resulted in a complete sudden loss of pigment. Thus conditions, which are thought to cause a reduction in the oxidative enzyme system associated with pigmentation and conditions which theoretically favor an inhibition or reversal of melanin formation, appear to be available for further exploration.

Martin, Wisansky and Ansbacher (106, 107) have reported that p-aminobenzoic acid has some effect in changing the normal course of melanin formation *in vitro*, but these results are not interpretable on the basis of the nutritional effects reported for this compound. In this regard we should bear in mind that the amounts of p-aminobenzoic acid which have been reported to cure achromotrichia in rats and humans are out of all proportion to the amount likely to be obtainable in food. The reported curative effect on achromotrichia may well prove to be an artifact which will have no counterpart in normal nutrition. How or where the remaining achromotrichia compounds might fit into the above scheme is beyond the limits of supposition at present. From the accumulated evidence it would seem that pigmentation in general is in part subject to proper hormone function and this in turn may depend on the adequacy and balance of certain nutritive factors.

SUMMARY

The fact that graying has been induced in all species of animals studied and has been observed in human infants under a variety of dietary deficiency conditions would seem to indicate that graying, particularly during growth, is a characteristic manifestation of certain types of dietary deficiency. The intent in the great majority of experiments has been to develop a specific deficiency of only one pigmentation factor. There is little chance that such extreme specific dietary imbalances often occur among humans, but multiple deficiencies undoubtedly do occur. That graying is ever actually induced under ordinary conditions by mild chronic deficiencies of various B-complex and mineral elements has not been proved. Since genetic, hormonal, and dietary influences have all been shown to play a part in pigmentation processes, the etiology of graying in individual people is unavoidably obscure. The practice of ascribing human curative or prophylactic properties to single pigmentation factors, which have been found effective in animals, under highly specialized and controlled conditions, is not well founded. It is not certain that graying in adult humans is ever caused by a dietary deficiency and, if such were indeed the case, the deficiency would almost certainly involve the lack of more than one of the factors thus far shown to be needed for normal pigmentation in animals.

Because of genetic influences, graying in adult human beings is not considered a manifestation of unnatural physiologic change. The fact that graying occurs in children on extremely poor diets is of considerable interest, but it is not very helpful in assessing the situation with regard to adults. The above-cited work with human beings was carried out before the need for pteroylglutamic acid for normal pigmentation was established in animals. Most of the human studies involved pantothenic acid. It is therefore of interest that there now appears to be physiologic relationship between these factors. The apparent functional relationship between pteroylglutamic acid and the anti-pernicious anemia principle of liver is also of interest, particularly in view of the fact that liver extract has been reputed to have an effect on graying in isolated instances. When more is known about about the nutritional rôle of the various pigmentogentic factors and about their interactions in essential enzyme systems, further study of thier effects in graying in human beings may be indicated.

REFERENCES

1. Bloch, B. *Das Pigment. Handbuch d. Haut-und Geschlichtskraukheiten.* Berlin, 1927.
2. Danforth, C. H. *Physiol. Rev.* 19:94, 1939.
3. Urbach, E. *Skin Diseases, Nutrition and Metabolism.* New York: Grune and Stratton, 1946.
4. Willier, B. H. *Ann. Surg.* 116:598, 1942.
5. Oleson, J. J., C. A. Elvehjem and E. B. Hart. *Proc. Soc. Exper. Biol. and Med.* 42:283, 1939.
6. Lachat, L. L. *Science* 93:452, 1941.
7. Editorial. *J. Am. Med. Assoc.* 128:302, 1942.
8. Bacharach, A. L. *Food* 10:219, 1941.
9. Gibbs, O. S. *Science* 95:576, 1942.
10. Barahal, H. S. *Psychiatric Quart.* 14:786, 1940.
11. Stieda, L. *Deutsch. Med. Wchnschr.* 36:1484, 1910.
12. *J. Am. Med. Assoc.* 57:1618, 1911.
13. Hrdlicka, A. *J. Am. Med. Assoc.* 118:918, 1942.

14. *Nutrition Rev.* 1:365, 1943; 2:90, 1944; 4:84, 1946.
15. NIEMES, P. AND L. WACKER. *Arch. Exp. Path. and Pharm.* 93:241, 1922.
16. HARTWELL, G. A. *Biochem. J.* 17:547, 1923.
17. BAKKE, A., V. ASCHEHOUG AND C. ZBINDEN. *R. Acad. Sci.* 191:1157, 1930.
18. KEIL, H. L. AND V. E. NELSON. *J. Biol. Chem.* 93:49, 1931.
19. GORTER, F. J. *Nature* 136:185, 1935; *Ziet. F. Vitaminforsch.* 4:277, 1934.
20. SJOLLEMA, B. *Biochem. Z.* 295:372, 1938.
21. STIRN, F. E., C. A. ELVEHJEM AND E. B. HART. *J. Biol. Chem.* 109:347, 1935.
22. LUTZ, R. E. *J. Ind. Hyg.* 8:177, 1926.
23. FREE, A. H. *Proc. Soc. Exp. Biol. and Med.* 44:371, 1940.
24. MORGAN, A. F., B. B. COOK AND H. G. DAVISON. *J. Nutrition* 15:27, 1938.
25. LUNDE, G. AND H. KRINGSTAD. *Avk. Norske Vid.-Akad.* Oslo: I. Mat. Nat. Kl., Nr. 1, 1938.
26. LUNDE, G. AND H. KRINGSTAD. *Zeit. f. Physiol. Chem.* 257:201, 1939.
27. MORGAN, A. F. AND H. D. SIMMS. *J. Nutrition* 19:233, 1940.
28. MORGAN, A. F. AND H. D. SIMMS. *J. Nutrition* 20:627, 1940.
29. LUNDE, G. AND H. KRINGSTAD. *Naturwissenshaften* 27:755, 1939.
30. LUNDE, G. AND H. KRINGSTAD. *J. Nutrition* 19:321, 1940.
31. GROODY, T. C. AND M. E. GROODY. *Science* 95:665, 1942.
32. GYÖRGY, P., C. E. POLING AND Y. SUBBAROW. *J. Biol. Chem.* 132:789, 1940.
33. GYÖRGY, P. AND C. E. POLING. *Science* 92:202, 1940.
34. GYÖRGY, P. AND C. E. POLING. *Proc. Soc. Exp. Biol. and Med.* 45:773, 1940.
35. NIELSEN, E., J. J. OLESON AND C. A. ELVEHJEM. *J. Biol. Chem.* 133:637, 1940.
36. UNNA, K. AND W. L. SAMPSON. *Proc. Soc. Exp. Biol. and Med.* 45:309, 1940.
37. DIMICK, M. K. AND A. LEPP. *J. Nutrition* 20:413, 1940.
38. WILLIAMS, R. R. *Science* 92:561, 1940.
39. FROST, D. V., F. P. DANN AND R. C. MOORE. *Proc. Soc. Exp. Biol. and Med.* 46:507, 1941.
40. ANSBACHER, S. *Science* 93:164, 1941.
41. MARTIN, G. J. AND S. ANSBACHER. *J. Biol. Chem.* 138:441, 1941.
42. EMERSON, G. A. *Proc. Soc. Exp. Biol. and Med.* 47:448, 1941.
43. UNNA, K., G. V. RICHARDS AND W. L. SAMPSON. *J. Nutrition* 22:553, 1941.
44. HENDERSON, L. M., J. M. MCINTIRE, H. A. WAISMANN AND C. A. ELVEHJEM. *J. Nutrition* 23:47, 1942.
45. DANN, F. P., R. C. MOORE AND D. V. FROST. *Fed. Proc. Am. Soc. Exp. Biol.* 1:107, 1942.
46. ANSBACHER, S. *Vitamins and Hormones*, Vol. 2. New York: Academic Press, 1944.
47. PAVCEK, P. L. AND H. M. BAUM. *Proc. Soc. Exp. Biol. and Med.* 47:271, 1941.
48. OWENS, H. S., M. TRAUTMAN AND E. WOODS. *Science* 93:406, 1941.
49. WOOLLEY, D. W. *Proc. Soc. Exp. Biol. and Med.* 46:565, 1941.
50. PAVCEK, P. L. AND H. M. BAUM. *Science* 93:502, 1941.
51. RALLI, E. P., D. H. CLARKE AND E. KENNEDY. *J. Biol. Chem.* 141:105, 1941.
52. MCGINNIS, J., L. C. NORRIS AND G. F. HEUSER. *J. Biol. Chem.* 145:341, 1942.
53. MARTIN, G. *Proc. Soc. Exp. Biol. and Med.* 51:353, 1942.
54. WRIGHT, L. D. AND A. D. WELCH. *J. Nutrition* 27:55, 1944.
55. FROST, D. V. AND F. P. DANN. *J. Nutrition* 27:355, 1944.
56. FROST, D. V., F. P. DANN AND F. C. MCINTIRE. *Proc. Soc. Exp. Biol. and Med.* 61:65, 1946.
57. LILLIE, R. J. AND G. M. BRIGGS. *Poultry Science* 26: 475, 1947.
58. EMERSON, G. A. AND H. M. EVANS. *Proc. Soc. Exp. Biol. and Med.* 46:655, 1941.
59. MOHAMMAD, A., O. H. EMERSON, G. A. EMERSON AND H. M. EVANS. *J. Biol. Chem.* 133:17, 1940.
60. UNNA, K. AND G. V. RICHARDS. *J. Nutrition* 23:545, 1942.
61. SCHWARZ, K. *Z. Physiol. Chem.* 275:245, 1942.
62. SCHWARZ, K. *Z. Physiol. Chem.* 275:232, 1942.
63. MILLS, R. C., J. H. SHAW, C. A. ELVEHJEM AND E. B. HART. *Proc. Soc. Exp. Biol. and Med.* 45:482, 1940.

64. Briggs, G. M., T. D. Luckey, R. C. Mills, C. A. Elvehjem and E. B. Hart. *Proc. Soc. Exp. Biol. and Med.* 52:7, 1943.
65. Emerson, G. A. and J. C. Keresztesy. *Proc. Soc. Exp. Biol. and Med.* 51:358, 1942.
66. Daft, F. S., L. L. Ashburn and W. H. Sebrell. *Science* 96:321, 1942.
67. Nielsen, E. and C. A. Elvehjem. *J. Biol. Chem.* 145:713, 1942.
68. Fritz, J. C., J. H. Hooper, J. L. Halpin and H. P. Moore. *J. Nutrition* 31:387, 1946.
69. Brandaleone, H., E. Main and J. M. Steele. *Am. J. Med. Sci.* 208:315, 1944.
70. Sieve, B. F. *Science* 94:257, 1941.
71. Sieve, B. F. *South. Med. Surg.* 104:35, 1942.
72. Kerlan, J. and R. P. Herwick. *J. Am. Med. Assoc.* 123:391, 1943.
73. Vorhaus, M. G., M. L. Gompertz and A. Feder. *Am. J. Digest Dis.* 10:45, 1943.
74. Brandaleone, H., E. Main and J. M. Steele. *Proc. Soc. Exp. Biol. and Med.* 53:47, 1943.
75. Nicholls, L. *Lancet.* 201, Aug. 10, 1946.
76. Peña-Chavarria, A., L. Goldman, C. Saenz-Herrera and E. Cordero-Carvajal. *J. Am. Med. Assoc.* 132:570, 1946.
77. Williams, C. D. *Arch. Dis. Childhood* 8:423, 1933.
78. Trowell, H. C. *Arch. Dis. Childhood* 12:193, 1937.
79. Gillman, T. and J. Gillman. *Arch. Int. Med.* 76:63, 1945.
80. Gillman, T. and J. Gillman. *J. Am. Med. Assoc.* 129:12, 1945.
81. Butcher, E. O. and R. A. Richards. *Endocrinology* 25:787, 1939.
82. Mushett, C. W. and K. Unna. *J. Nutrition* 22:565, 1941.
83. Ralli, E. P. and I. Graef. *Endocrinology* 32:1, 1943.
84. Ralli, E. P. and I. Graef. *Endocrinology* 37:252, 1945.
85. Ralli, E. P. and I. Graef. *Am. J. Physiol.* 140:713, 1943.
86. Gerstl, B., B. Lustig and A. R. Goldfarb. *Science* 96:447, 1942.
87. Forbes, T. R. *Endocrinology* 30:465, 1942.
88. Rothman, S. *J. Lab. and Clin. Med.* 27:687, 1942.
89. Raper, H. S. *J. Chem. Soc.* 1:125, 1938.
90. Bloch, B. *Z. Physiol. Chem.* 98:226, 1917.
91. Figge, F. H. J. *Proc. Soc. Exp. Biol. and Med.* 46:269, 1941.
92. Arnow, L. E. *J. Biol. Chem.* 120:151, 1937.
93. Rothman, S. *J. Investig. Dermatology* 5:61, 1942.
94. Rothman, S. *J. Investig. Dermatology* 5:67, 1942.
95. Hogeboom, G. H. and M. H. Adams. *J. Biol. Chem.* 145:273, 1942.
96. Onslow, H. *Proc. Roy. Soc. Lond.* B89:36, 1915.
97. Pugh, C. E. *Biochem. J.* 27:475, 1933.
98. Richter, C. P. and K. H. Clisby. *Proc. Soc. Exp. Biol. and Med.* 48:684, 1941.
99. Bernheim, F. and W. L. C. Bernheim. *J. Biol. Chem.* 145:213, 1942.
100. Keilin, D. and T. Mann. *Proc. Roy. Soc.* B125:187, 1938.
101. Kubowitz, F. *Biochem. Ztschr.* 299:32, 1938.
102. Dalton, H. R. and J. M. Nelson. *J. Am. Chem. Soc.* 60:3085, 1938.
103. Oettel, H. *Arch. Exp. Path. and Pharm.* 183:319, 1936.
104. Schroeder, H. *Klin. Wschr.* 13:553, 1934.
105. Goodman, M. H. *Arch. Derm. and Syph.* 40:76, 1939.
106. Martin, G. J., W. A. Wisansky and S. Ansbacher. *Proc. Soc. Exp. Biol. and Med.* 47:26, 1941.
107. Wisansky, W. A., G. J. Martin and S. Ansbacher. *J. Am. Chem. Soc.* 63:1771, 1941.